EZH2 AND NF-κB CROSSTALK IN BREAST CANCER

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Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Lee Shuet Theng

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Summary

Basal-like breast cancer (BLBC) and luminal breast cancer are two major subtypes of breast cancer and BLBC represent the more aggressive subtype compared to luminal breast cancer. This observation is associated with higher expression of EZH2 and constitutive activation of NF-κB pathway in BLBC. In this study, we sought to dissect the crosstalk between EZH2 and NF-κB under these two cellular contexts.

From our genome-wide mRNA expression profiling in a BLBC cell line, MDA-MB-231, EZH2 was found to positively modulate NF-κB-mediated inflammatory responses. This relationship was validated by a series of cell-based assays. We examined the DNA recruitment of EZH2 and NF-κB on the promoter of NF-κB target genes as well as the changes of the target genes expression. EZH2 was found to exert a positive role in regulating DNA binding activity of RelA and RelB, and accordingly upregulate the expression levels of NF-κB target genes. Using co-immunoprecipitation and *in vitro* pull down assay, we demonstrated that EZH2 could physically interact with RelA and RelB forming a ternary complex. These interactions did not require SET domain of EZH2, suggesting a novel function of EZH2 independent of its SET-dependent histone methylation activity.

The importance of this crosstalk was further demonstrated by analyzing EZH2/RelA/RelB coregulated genes in terms of their association with metastases in different breast cancer subtypes. Strikingly, there was a set of 12 genes, which were consistently expressed higher in BLBC or ER-negative breast cancer tissues and showed significant association with lung and brain metastases. This outcome revealed a potential role of EZH2/RelA/RelB crosstalk in promoting invasion and metastasis of aggressive breast cancer.

Unlike ER-negative BLBC cells, ER-positive luminal breast cancer cells showed reduced level of RelB and concurrently exhibit high level of ER as well as its cofactors such as FOXA1 and GATA3. Under this cellular context, EZH2 was found to function as a negative regulator of NF-κB target genes expression. This regulation was revealed to be

dependent on the canonical H3K27 trimethylation activity of EZH2, potentially recruited by ER to the promoter of NF-κB target genes *IL8* and *IL6*. Interestingly, the ectopic overexpression of RelB in ER-positive luminal breast cancer cell line, MCF7, could partially revert the function of EZH2 to become the transactivator of NF-κB target gene, *IL6*. These observations suggest that the presence of RelB and ER as possible crucial determinants of the functionality of EZH2 in regulating NF-κB gene network.

Taken together, this study proposed a model highlighting a dual-function of EZH2 in modulating NF-κB network depending on cellular context. Importantly, the balance of ER and RelB expression could possibly be the major factors in determining the mode of EZH2 regulation on NF-κB network.

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List of Abbreviations

3D	Three dimensional
AR	Androgen receptor
BIRC3	Baculoviral IAP repeat containing 3
BLBC	Basal-like breast cancer
BMI1	B lymphoma Mo-MLV insertion region 1
BRCA1/2	Breast cancer 1/2, early onset
CBX1	Chromobox homologues
CCNB1	Cyclin B1
CDK1/2	Cyblin-dependent kinase 1/2
ChIP	Chromatin immunoprecipitation
CK5/6/7/8/17/18/19	Cytokeratin 5/6/7/8/17/18/19
co-IP	Co-immunoprecipitation
COX1/2	Cyclooxygenase 1/2
CSC	Cancer stem cell
CTC	Circulating tumor cells
DAB2IP	DAB2 interacting protein
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
DTT	Dithiothreitol
DZNep	3-Deazaneplanocin A
EDTA	Ethylenediamine tetra-acetic acid
EED	Embryonic ectoderm development
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
EZH2	Enhancer of zeste homologue 2
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FOXA1	Forkhead box A1
GATA3	GATA binding protein 3
GFP	Green fluorescence protein
GST	Glutathione S-transferase
H3K27	Histone 3 Lysine 27
H3K27me3	Histone 3 Lysine 27 trimethylation
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HMEC	Human mammary epithelial cell
IKK	IκB kinase
IL1	Interleukin 1

IL6	Interleukin 6
IL8	Interleukin 8
IPA	Ingenuity pathway analysis
IκBα	NF-κB inhibitor α
JMJD3	Jumonji D3
LTβ	Lymphotoxin β
MBP	Maltose-binding protein
MDB	Methyl-CpG binding domain proteins
miRNA	Micro RNA
mRNA	Messenger RNA
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate-buffered saline
PcG	Polycomb group
pCR	Pathological complete response
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PRC1	Polycomb repressor complex 1
PRC2	Polycomb repressor complex 2
PRE	Polycomb response element
PTGS2	Prostaglandin-endoperoxide synthase 2
PVDF	Polyvinyllidene difluoride
RTK	Receptor tyrosine kinase
RT-PCR	Real-time polymerase chain reaction
SAA1	Serum amyloid A1
SDS	Sodium dodecyl sulphate
siRNA	small-interfering RNA
SUZ12	Suppressor of zeste 12
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TSS	Transcription start site
UTR	Untranslated region
WT	Wild type
Xist	X inactivation specific transcript

CHAPTER 1: INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common cancer in women and it is the second leading cause of cancer death in women after lung cancer. Breast is constituted of ducts, lobules, adipose, connective and lymphatic tissues. Breast cancer is normally arised from ductal or lobular tissues (Figure 1.1A). At the initial stage of tumor development, the tumor mass is confined in the ductal or lobular structure, it is termed *in situ* carcinoma. *In situ* carcinoma usually would result in a good clinical outcome as long as it is surgically removed. On the other hand, when the cells become invasive and infiltrate to the adjacent tissue, there would be a high possibility that it will metastasize to other organs and often results in poor prognosis (AmericanCancerSociety, 2011).

Breast cancer could also be classified into basal-like and luminal subtypes based on molecular expression profiling (Perou *et al.*, 2000). These two subtypes are generally viewed as different disease entities and have very different intrinsic gene expression patterns. Mainly, there are two cell types that exist in the ductal and lobular tissues, namely luminal and myoepithelial (Figure 1.1B). Both basal and luminal breast cancer cells are proposed to be originated from the luminal lineages but arised at different stages of development. Basal-like breast cancer was believed to develop from the luminal progenitor cells that are more pluripotent and mesenchymal as compared to the luminal breast cancer that was proposed to develop from more differentiated luminal stage (Prat and Perou, 2009). Thus, basal-like breast cancer retains the expression of genes that are present in the myoepithelial lineage that was branched right before or at the stage of luminal progenitor. On the other hand, luminal breast cancer which develops later has lost the traits of myoepithelial cells and instead gained the expression of the genes that is similar to the differentiated luminal cells.

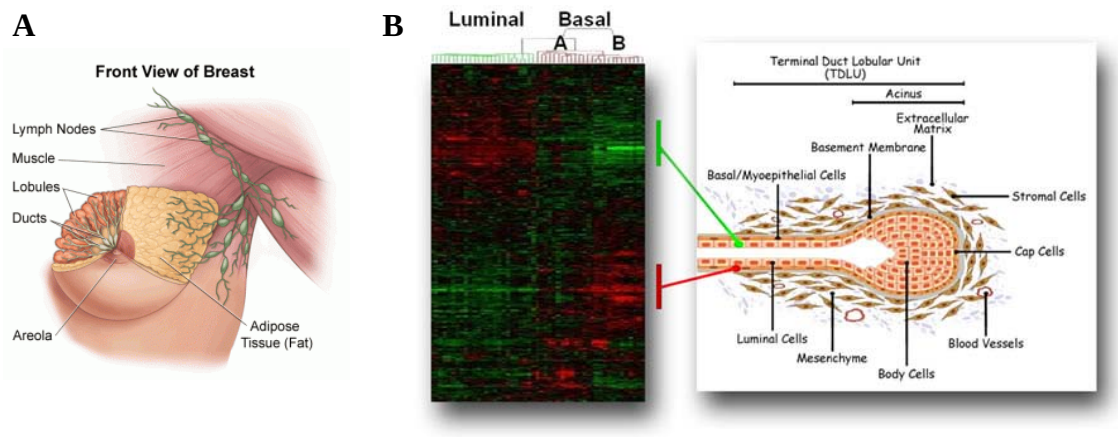

Figure 1.1 Structure of breast and subtype classification

A. Anatomy structure of breast organ. (Adapted from: http://www.surgical-blog.com/breast-cancer-about-breast-cancer-breast-cancer-risk-and-symptoms/)
B. Luminal and basal-like breast cancer classification was defined by the expression profiles of the intrinsic genesets. (Adapted from: http://www.lbl.gov/Science-Articles/Archive/sabl/2007/Apr/bc.html)

The heterogeneity of breast cancer was intensively being studied by the advancement of gene expression profiling using high throughput technologies. Basal-like and luminal breast cancer are now further subcategorized into several subgroups based on the expression of specific genes (Lehmann *et al*., 2011). In addition, more breast cancer subtypes that are not categorized to luminal and basal-like also emerged, for instance claudin-low breast cancer (Table 1.1 and Table 1.2) (Peddi *et al*., 2012). In this thesis, my focus will be on the basal-like and luminal breast cancer in general.

Intrinsic subtypes of breast cancer	Characteristics
Luminal A	High level expression of ER and ER-associated genes, associated with a favorable clinical outcome.
Luminal B	Low level expression of ER and ER-associated genes, associated with a higher tumor cell proliferation rate and a worse clinical outcome compared to the luminal A subtype.
HER-2 Enriched	High level expression of HER2 and GRB7, associated with a poor outcome before the era of HER2-targeted agents.
Basal-like	Positive for the expression of basal cytokeratin but negative for the expression of luminal- and HER2-related genes, associated with a high tumor cell proliferation rate and a poor clinical outcome.
Normal-like	Similar expression compared to normal breast, suspicious for normal cell contamination.
Claudin-low	Lack the expression of claudin proteins that are implicated in cell-cell adhesion, but high expression of EMT and putative stem cell markers, associated with ER and HER2 negativity but low in basal cytokeratin expression.

Table 1.1: Intrinsic subtypes of breast cancer(Peddi *et al*., 2012)

Subtype	Gene expression profile
Basal-like 1 (BL-1)	High in the expression of genes involved in cell cycle progression, cell division, and DNA damage response pathways.
Basal-like 2 (BL-2)	High in the expression of genes involved in cell cycle progression, cell division, and growth factor signaling.
Immunomodulatory (IM)	High in the expression of genes involved in immune processes and cell signaling.
Mesenchymal (M)	High in the expression of genes involved in motility and extracellularmatrix.
Mesenchymal stem-like (MSL)	High in the expression of genes involved in motility, extracellular matrix, and growth factor signaling; consistent with claudin-low Intrinsic subtype.
Luminal androgen receptor (LAR)	High in the expression of genes involved in hormonally regulated pathways.

Table 1.2: Six subtypes of triple negative breast cancer based on gene expression profiling. (Peddi *et al*., 2012)

1.1.1 Basal-like breast cancer (BLBC)

About 15% of breast cancer is categorized as basal-like breast cancer (BLBC) (Rakha *et al.*, 2008). BLBC could be distinguished from luminal breast cancer by immunohistochemistry staining of the markers that are expressed specifically in basal-like cells, for instance keratin 5, keratin 6, and keratin 17. Another common characteristic of basal-like breast cancer cells is the lack of expression of several surface receptors like estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), and in most of the cases progesterone receptor (PR). Due to the negativity of the expression of these receptors, this subtype of breast cancer is also known as triple-negative breast cancer (TNBC) (Foulkes *et al.*, 2010). About 70-80% of BLBC cells are TNBC and more than 80% of TNBC belongs to BLBC. Furthermore, there are great similarities shared between TNBC and BLBC in terms of clinical prognosis and therapeutic responses (Badve *et al.*, 2011). In fact, many clinical trials that were supposedly performed in BLBC were carried out in patients stratified by triple-negativity status due to convenient and pragmatic purposes (Rakha *et al.*, 2008; Santana-Davila and Perez, 2010). Therefore, although it was reported that the overlap between these two breast cancer types is not complete, many papers still used BLBC and TNBC interchangeably. Concordantly, in the introduction, I will summarize the findings in BLBC and TNBC in a collective manner.

1.1.1.1 Aggressive phenotypes of BLBC

BLBC frequently occurs in young women especially from black and Hispanic races compared to other ethnic groups (AmericanCancerSociety, 2011). Patients with BLBC tend to have adverse prognosis and early relapse within five years after treatment (Fig 1.2) (Rakha *et al.*, 2008). The recurrent tumors are always found to be at the distal organs from breast, indicating the occurrence of metastatic events. Metastasis is an event when tumor cells

disseminate from its primary niche to other sites of the body to form secondary tumors (Nguyen *et al*., 2009). It happens when tumor progresses to higher grade and attain invasive and aggressive properties. Metastasis is the main cause that renders this disease to be incurable.

Clinical observation has revealed that different types of cancer could have preference towards the sites where they metastasize to, this phenomenon termed “organ tropisms” (Chu and Allan, 2012; Nguyen *et al*., 2009). For instance, breast cancer preferentially metastasizes to lymph nodes, lungs, brain, liver, and bone; whereas prostate and colorectal cancers tend to spread to bone and liver, respectively. Interestingly, it was reported that different subtypes of breast cancer could too have different preference towards sites of metastasis: BLBC has higher propensity to spread to brain, lung, and liver whereas luminal breast cancer has higher propensity to spread to bone, liver, and lung (Foulkes *et al*., 2010).

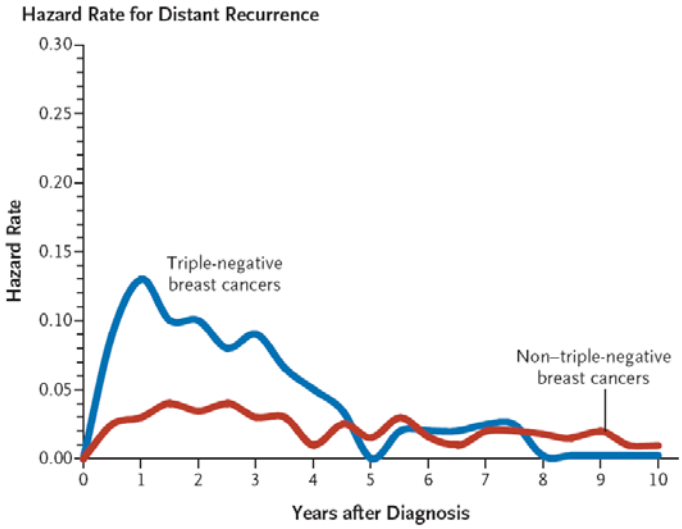

Figure 1.2 Hazard rates for distant recurrence of TNBC and non-TNBC breast cancer. (Foulkes *et al*., 2010)

Two factors were proposed to influence the aggressiveness of BLBC, the mesenchymal phenotype and the enriched population of cancer stem cells. Under normal circumstances, epithelial cells are well-organized and reside within the basement membrane

with regular apical basolateral polarity. During cancer progression, the cancer cells would undergo a process called epithelial-mesenchymal transition (EMT) (Kang and Massague, 2004; Thiery, 2002), in which the cells would gradually lose the expression of cell-cell junction molecules (e.g., E-Cadherin) , which results in the disorganization of the cell polarity and the gain of cell motility (Figure 1.3). The cells would then invade out from the primary tumor site, migrate and colonize distal organs to form secondary metastases. It was discovered that EMT features happen more frequently in BLBC (Sarrio *et al*., 2008) Many of the BLBC cell lines are locked in mesenchymal state with characteristics which are highly motile, invasive, lack of cell-cell adhesion junction and often appear to have spindle-shaped morphology.

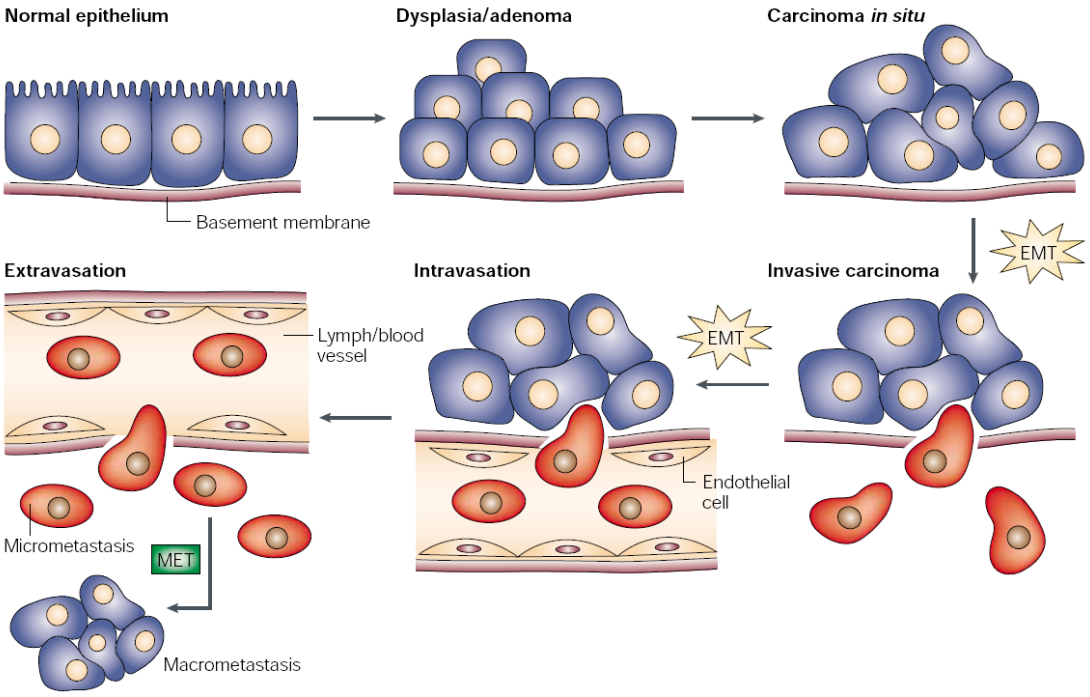

Figure 1.3 Epithelial to mesenchymal transition in cancer progression (Thiery, 2002)**.**

Another factor that contributes to the aggressiveness of BLBC is the high proportion of cancer stem cells (CSC) in BLBC (Honeth *et al*., 2008). CSC represents a population of cells that is able to initiate tumor formation and subsequent tumor maintenance *in vivo*. As CSC is found to be resistant to chemotherapy, it has been regarded to be the main culprit of

tumor relapse. The first isolation of CSC in breast tumors was achieved by Al-Hajj *et al.* via fluorescence-activated cell sorting based on the expression of the surface markers $CD44^{+}/CD24^{-/low}$.(Al-Hajj *et al*., 2003) The isolated cells were demonstrated to be able to generate tumor *in vivo* with as few as 100 cells injected. Strikingly, BLBC has been reported to be enriched in $CD44^{+}/CD24^{-/low}$ cell population (Honeth *et al*., 2008; Park *et al*., 2010). This could serve as the cause of clinical observation that BLBC tends to have early relapse after chemotherapy due to the presence of high fraction of CSC.

CSC was shown to have increased metastatic propensity *in vitro* and *in vivo*, albeit mechanism remains unclear (Chu and Allan, 2012). Intriguingly, it was suggested that EMT could accelerate the formation of CSC (Floor *et al*., 2011; Morel *et al*., 2008). A recent paper even proposed that EMT cells and CSC are overlapped and among the EMT cells disseminated from the tumor only the most competent CSC will eventually succeed to metastasize (Floor *et al*., 2011). Although the relationship between EMT and CSC is complex and perplexing, their association with cancer metastasis is evident. As BLBC is enriched in both CSC and EMT phenotypes, propensity to metastasize is also unquestionable.

1.1.1.2 Pathways driving BLBC oncogenicity

Cancer development and progression often involves the activation of oncogenic pathways and inactivation of tumor suppression pathways by genetic or epigenetic changes. Different cancer types may have dependency on the dysregulation of different signaling pathways. In BLBC, several pathways are known to be dysregulated and contributed to its progression, for instance silencing of ER and BRCA1, overexpression or activation of EGFR, VEGFR, SRC, PI3K, and NF-κB pathways.

As mentioned earlier, more than 80% of BLBC is TNBC, lacking the expression of ER, PR and HER2. In fact, many studies have demonstrated that the absence of ER is one major factor of the aggressiveness of BLBC (Fearon, 2003; Rochefort *et al*., 1998). Exogeneous expression of ER in BLBC was shown to reduce the invasiveness and

aggressiveness of the cells . The mechanism behind ER-mediated suppression of cancer invasiveness is not largely understood, although several findings have demonstrated that ER negatively regulates the expression of the components in NF-κB pathways, including RelB, IL6, and IL8 (Freund *et al*., 2003; Freund *et al*., 2004; Stein and Yang, 1995; Wang *et al*., 2007). NF-κB pathway was long known to be constitutively activated in BLBC, and it is shown to be associated with the aggressiveness of this subtype of breast cancer (Gionet *et al*., 2009; Huber *et al*., 2004; Karin and Greten, 2005). However, the mechanism underlying the constitutive activation of NF-κB pathway is not well understood.

EGFR is frequently overexpressed in BLBC (Dent *et al*., 2007). In some studies, EGFR is even proposed to be one of the molecular markers of BLBC apart from the negativity of ER, PR, HER2 and the expression of basal keratins (Shao *et al*., 2011; Siziopikou and Cobleigh, 2007). Recent reports indicated that the overexpression EGFR protein level is largely due to gene amplification in BLBC (Gumuskaya *et al*., 2010; Shao *et al*., 2011). As a consequence of EGFR overexpression, EGFR signaling was found to be overactivated in BLBC. Besides promoting the survivability of cancer cells, the activation of EGFR pathway in BLBC was shown to enhance the mesenchymal phenotypes of this cancer subtype (Ueno and Zhang, 2011).

Tumor cells are fast growing cells but the growth of tumor mass is restricted by the availability of nutrients and oxygen. Thus, when the tumor mass grows to a certain size, typically 1-2mm (McDougall *et al*., 2002), tumor cells would secrete growth factors like fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) that will induce a process called angiogenesis. Angiogenesis is one crucial process during tumor progression, involving the development of blood vessels at the site where tumor mass grows. The blood capillaries that grow into the tumor mass would nourish the tumor cells to support further tumor growth. Besides nutrients and oxygen supply, angiogenesis was indicated to promote metastasis. Cancer cells that gain metastatic potential could invade into the blood vessels and enter the blood circulation and turn out to be circulating tumor cells (CTC). CTC

was demonstrated to correlate with the development of secondary metastatic tumors (Cristofanilli *et al*., 2004).

SRC is a non-receptor tyrosine kinase, which would relay phosphorylation signaling upon activation by growth factor receptor. As consequences of dysregulated SRC activation, the cells would gain oncogenic responses such as increase in cell proliferation, survivability, angiogenicity, and motility (Gelman, 2011; Wheeler *et al*., 2009). It was reported that SRC is overactivated in BLBC, and BLBC cells were found to be more susceptible for SRC inhibitors (Finn *et al*., 2011; Tryfonopoulos *et al*., 2011), implicating the dependency of BLBC on the dysregulated SRC activity.

Besides the abovementioned factors/pathways that contribute to the aggressiveness of BLBC, there are also pathways that are known to be governing the survivability or proliferation specifically in BLBC subtype. BRCA1 is a tumor suppressor, which functions to repair double-stranded breaks in DNA for instance during the events of homologous recombination. BRCA1 expression could be silenced by genetic (inactivating mutation) or epigenetic (promoter hypermethylation and microRNA regulation) mechanisms (Rakha *et al*., 2008; Rice *et al*., 1998; Shen *et al*., 2009; Shen *et al*., 2010). Loss of the expression of BRCA1 leads to error-prone DNA repair, thereby increasing the risk of genetic mutations and hence predisposed to cancer development. It was discovered that breast cancer that arises from BRCA1-deficient cells shares great similarity to BLBC in terms of clinical characteristics and intrinsic gene expression (Santana-Davila and Perez, 2010). Although it is unclear of the cause and effect relationship between BRCA1-associated breast cancer and BLBC, some reports have suggested that BLBC is more tolerable to the deficiency of BRCA1 (Bryan *et al*., 2006; Dabbs *et al*., 2006) while some other reports have suggested that loss of BRCA1 leads to the development of cancer with more stem-cell like properties (Foulkes, 2004; Furuta *et al*., 2005), which is in concordance with the phenotype of BLBC.

When BLBC cells were defined based on the triple-negativity status, researchers discovered that there is a subgroup of BLBC segregated with luminal breast cancer based on expression profiling despite of the absence of ER, PR, and HER2 (Doane *et al*., 2006). Subsequently, it was demonstrated that the expression of androgen receptor (AR) causes the similarity of this BLBC subgroup with luminal breast cancer, therefore this subtype of breast cancer is named as luminal androgen receptor (LAR). Investigators reported that the expression of AR in LAR renders the proliferative advantage of this cancer subtype.

In addition to the aforementioned pathways that are more specifically being activated/inactivated in BLBC/TNBC compared to other cancer subtypes, many other oncogenic pathways are also revealed to play essential roles in promoting breast cancer in general, including both BLBC and luminal breast cancer (Cleator *et al*., 2007; Santana-Davila and Perez, 2010). For example, PI3K/AKT/mTOR and Ras/MEK/MAPK pathways were known to promote survivability and proliferation in breast cancer. Ordinarily, the pathways that are dysregulated are not mutually exclusive, multiple oncogenic pathways could be turned on and multiple tumor suppressor pathways could be turned off simultaneously and contributed to oncogenesis.

1.1.1.3 Current therapy of BLBC

Most BLBC lacks the expression of ER, PR, and HER2, rendering this cancer subtype irresponsive to receptor targeted therapy. Hence, chemotherapy is the mainstay therapeutic option for the treatment of this subtype of breast cancer (Berrada *et al*., 2010). Chemotherapeutic drugs are also known to be cytotoxic drugs that attempt to kill fast growing cells. In the case of neoplasia, chemodrugs are effective in the treatment by exploiting the fact that the tumor cells proliferates much faster than normal cells. There are several types of chemotherapeutic agents which are frequently used in neoadjuvant or adjuvant therapy of BLBC (Cleator *et al*., 2007; Santana-Davila and Perez, 2010), including (i) anthracyclines -

compounds extracted from Streptomyces bacteria, normally act by intercalating DNA/RNA and interfere with cell replication (e.g., doxorubicin); (ii) taxanes – compounds produced by plants of the genus Taxus, act by disrupting microtubule function (e.g., paclitaxel); (iii) platinum agents – DNA damaging agents, act by inducing DNA repair mechanisms and in turn inducing apoptosis when repair is impossible (e.g., cisplatin).

Among different subtypes of breast cancer, BLBC patients were revealed to benefit most from chemotherapy with higher pathological complete response (pCR), an indication of the complete recession of detectable tumor mass (Foulkes *et al*., 2010). However, the overall survival rate of BLBC patients does not appear to be favorable due to early tumor relapse (Dent *et al*., 2007). The recurrent BLBC tumors are usually metastatic and appear more aggressive. As a result, less than 30% of the women with BLBC are able to survive five years. Therefore, intensive research efforts are focused on the identification of druggable targets in TNBC and develop better pharmaceutical strategies in BLBC treatment.

Thus far, molecular targeted therapy has limited success in the treatment of BLBC. One example is poly-(ADP ribose) polymerase (PARP) inhibitor which was once believed to be a promising targeted drug in treating BLBC. About 20% of BLBC was reported to have BRCA1-deficiency, either via gene mutation or gene underexpression. As mentioned earlier, BRCA1 is responsible for homologous DNA repair. Studies have reported that tumors with BRCA1 deficiency showed synthetic lethality with PARP inactivation, which would otherwise function to repair DNA through base-excision (Glendenning and Tutt, 2011). However, during phase III clinical trial of Iniparib (PARP inhibitor), no favorable outcome was observed in BLBC treatment albeit the mechanism of resistance is still unknown (Fojo *et al*., 2011; Guha, 2011).

Another example of targeted therapy is EGFR inhibitors (Harari, 2004), which encompass EGFR-specific antibodies (eg. Cetuximab) and small molecule inhibitors (eg. Gefitinib and Erlotinib). The rationale behind EGFR-targeted therapy is based on the findings

that more than 50% of TNBC harbors EGFR overexpression and hyperactivation. EGFR is a receptor tyrosine kinase (RTK) that is responsible for the activation of multiple downstream oncogenic kinase signalings such as AKT and ERK upon activation by cytokines like EGF (Foley *et al*., 2010). These signalings lead to cancer progression for instance increase in cell proliferation, invasiveness, and survivability. Nevertheless, similar to the case of PARP inhibitor, EGFR inhibitors showed limited favorable response during clinical trials (Hudis and Gianni, 2011). Many possible mechanisms was reported including mutation of EGFR that affects the binding of the inhibitors, nuclear localization of EGFR that renders inefficient cell surface binding of Cetuximab (Wheeler *et al*., 2010). The disappointing clinical outcomes of EGFR inhibitors underscore the need of a revised EGFR targeted strategy.

In addition to these approaches, other oncogenic factors that were known to be overactivated in BLBC were also being targeted (Berrada *et al*., 2010; Peddi *et al*., 2012). For instance, SRC inhibitor (Dasatinib), VEGF inhibitor (Bevacizumab), and AR inhibitor (Bicalutamide) are under clinical trials to access the efficacy in BLBC/TNBC therapy.

1.1.2 Luminal breast cancer

Majority (75%-80%) of breast cancer is characterized as luminal breast cancer. Luminal breast cancer cells are normally characterized by the expression of luminal cytokeratin for instance CK7, CK8, CK18, and CK19 (Perou *et al*., 2000). In addition to these markers, it is also generally recognized that luminal breast cancer cells express PR, ER and its associated cofactors like FoxA1 and GATA3 (Badve and Nakshatri, 2009).

Clinically, luminal breast cancer is further subcategorized into two groups, namely luminal A and luminal B subtypes. These two subtypes could be distinguished by proliferative signatures such as CCNB1, MK167 and MYBL2, which expressed higher in luminal B subtype (Cheang *et al*., 2009). It was also suggested that high expression of HER2 and Ki67 (another proliferative marker with immunohistochemistry antibody available), also allows the distinction of luminal B subtype from luminal A.

1.1.2.1 Phenotypes

Most of the luminal breast cancer cells appear epithelial with high expression of cell adhesion molecules like E-cadherin (Fearon, 2003). As a result, luminal cells are generally less invasive than BLBC. Similarly, by clinically comparing luminal breast cancer to BLBC, the former is less aggressive with lower frequency of tumor recurrence in five years after surgery (Andre and Pusztai, 2006). Furthermore, luminal breast cancer patients would normally experience local tumor recurrence before distant recurrence. Hence, local recurrence in luminal breast cancer could serves as an observation predictive of distal metastasis. When comparison is made between the two luminal subtypes, it was observed that luminal B is more proliferative and invasive than luminal A.

Interestingly, it was discovered that there are discordance of ER, PR, and HER2 status between the primary and recurred metastatic breast cancer (Arslan *et al*., 2011). Most of the time, these receptors are lost in relapsed metastatic tumors, particularly for the case of PR (Broom *et al*., 2009). It was suggested that most tumors represent a heterogeneous cell population. Although luminal breast cancer cells harbor the expression of ER, PR, and HER2, it was reported that more than 50% of luminal breast cancer consists of about 1% of ER-, PR-negative, and CK5-positive cells that resemble BLBC (Haughian *et al*., 2012). This subpopulation of cells is known as luminobasal cells. Thus, it was speculated that this luminobasal minor population is the culprit accountable for the resistance of receptor-targeted therapy (Kabos *et al*., 2011), which survives and relapses after such therapy.

1.1.2.2 Pathways driving luminal breast cancer oncogenicity

In past decades, research efforts in luminal breast cancer were focused on ER, PR, and HER2. Researchers found that ER positively regulates several aspects of luminal breast cancer especially cell survivability and proliferation (Ali and Coombes, 2000; Sommer and Fuqua, 2001). ER is a family of nuclear hormone receptor, which would translocate into the nucleus to regulate gene transcription upon activation of its ligands for instance, estrogen. It could either activate or suppress the expression of its target genes by binding to its co-activators or co-repressors. GATA3 and FoxA1 are two essential collaborators of ER (Badve and Nakshatri, 2009). Investigators reported that GATA3 and FoxA1 often serve as pioneering factors to aid ER loading to the DNA binding sites of its target genes. Loss of either GATA3 or FoxA1 was demonstrated to disrupt ER target gene regulation. Cyclin D1 and c-myc (Wang *et al*., 2011) are two well known examples of ER that promote cell proliferation and survival, respectively. Similar to ER, PR is also known to promote proliferation in breast cancer (Obr and Edwards, 2012), although the exact mechanism of how PR could do so is less well studied.

Perplexingly, ER and PR were shown to suppress luminal breast cancer progression to the advanced metastatic stage. One proposed mechanism is through ER suppression of Snail expression (Fearon, 2003), an EMT driver, which otherwise would repress E-cadherin expression. As a result, ER-positive breast cancer cells are usually associated with high expression of E-cadherin and adopt epithelial and non-invasive phenotypes. In addition, ER was also reported to repress RelB (Wang *et al*., 2007), a member of NF-κB that was proposed to function as a metastatic driver. On the other hand, how PR represses EMT was not well understood.

HER2 is implicated in luminal B subtype of breast cancer. HER2 was revealed to regulate a wide array of downstream signaling pathways including MAPK, PI3K, and STAT (Ross and Fletcher, 1998; Ross *et al*., 2003), that are known to promote proliferation, survival, and invasion during oncogenesis. Indeed, HER2 expression in luminal B breast cancer was suggested to be one of the factors causing luminal B to be more aggressive than luminal A breast cancer (Cheang *et al*., 2009).

1.1.2.3 Current therapy

As luminal breast cancer is largely dependent on ER for survival and proliferation, it is not inconceivable that the main approach of luminal breast cancer therapy is endocrine therapy targeting ER. There are two types of endocrine therapy targeting ER (Shao and Brown, 2004), (i) selective estrogen receptor modulators (SERM), act as ER antagonists (e.g., tamoxifen and raloxifene); (ii) aromatase inhibitors, act by inhibiting estrogen synthesis from androgen (e.g., anastrozole). In fact, the more commonly adopted therapy using tamoxifen has produced much efficacy in treating luminal breast cancer. Based on breast cancer statistics in USA, the breast cancer death rate in white American diverged significantly from African American starting from 1990s (Fig 1.4.). Two reasons were proposed to explain for this difference (Jatoi *et al*., 2003; Menashe *et al*., 2009): (i) greater usage of mammography by whites, thus enable early detection and surgical treatment ; (ii) introduction of tamoxifen,

which could benefit white American more as these patients tend to have luminal breast cancer (ER-positive) compared to black American whom often suffer from BLBC (ER-negative).

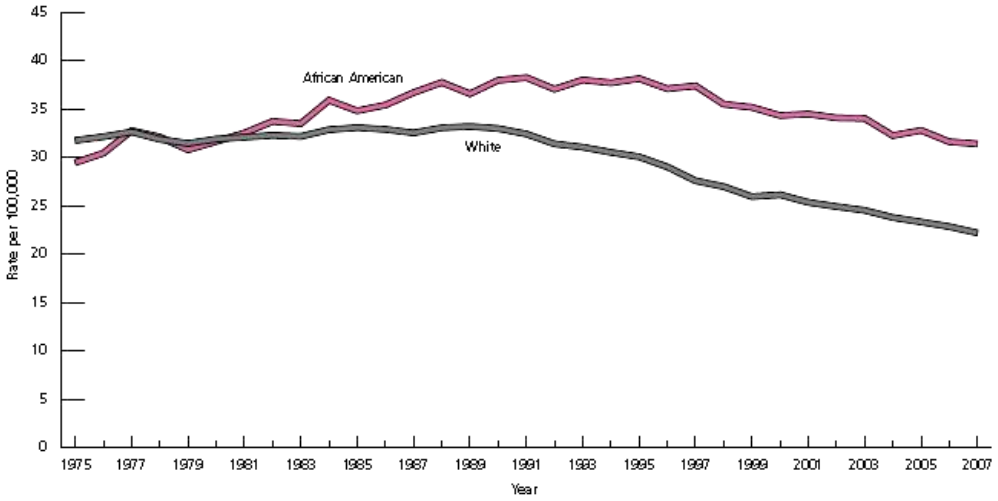

Figure 1.4 Trends of death rates in female breast cancer patients in United States. Modified from: (AmericanCancerSociety, 2011)

Nevertheless, in 2000s, a subset of patients was discovered to develop resistance towards tamoxifen. Soon after, it was revealed that the elevation of HER2 level in the treated luminal breast cancer could be accountable for the resistance (Shou *et al*., 2004). Therefore, investigators attempted to combine treatment of tamoxifen with trastuzumab (Romond *et al*., 2005), a HER2 inhibitor, and the effectiveness of this strategy was evident in the clinical outcomes.

Although HER2 expression is elevated in luminal B breast cancer, trastuzumab did not yield a satisfactory outcome clinically (Nguyen *et al*., 2008). A possible reason is that HER2 is only an auxiliary pathway driving the oncogenicity of this subtype of breast cancer. Many other oncogenic pathways activated in this cancer subtype appear to be resulted from the activation of various growth factor receptors. Hence, it was suggested that other kinases inhibitors for instance PI3K inhibitor could be a better candidate for the treatment (Loi, 2008) as these kinases lie at the convergent point downstream of the different growth factor receptors in the signaling pathways.

1.2 EZH2

In 1980s, enhancer of zeste (E(Z)) was first found to play a role in suppressing the differentiation of cells into specific tissues, thereby affecting the development of Drosophila melanogaster (Jones and Gelbart, 1990). In 1990s, researchers discovered that the human homolog of E(Z), named Enhancer of Zeste Homolog 2 (EZH2) is involved in the transcriptional repression of homeobox gene expression (Hobert *et al*., 1996), to maintain stemness and inhibit differentiation of the cells. It was found that EZH2 deficient mice are embryonic lethal, indicating its importance in early development. In adults, EZH2 remains essential as it was reported to be involved in the self-renewal of hematopoietic stem cells that would populate blood cells from myeloid and lymphoid lineages.

In addition, EZH2 was also shown to participate in X-chromosome inactivation. In female mammals (Plath *et al*., 2003), X-chromosome inactivation is a crucial event during development to achieve dosage compensation by inactivating one of the two copies of X-chromosomes. During the inactivation process, a non-coding RNA called X inactivation specific transcript (*Xist*) would be transcribed and bound to the inactivating X-chromosome. This facilitates the recruitment of PRC2 and subsequent H3K27 trimethylation, which would eventually lead to chromosome compaction and gene inactivation.

1.2.1 EZH2 and PRC2 complex

EZH2 is the catalytic component of polycomb repression complex 2 (PRC2) (Simon and Lange, 2008). The protein structure of EZH2 is illustrated in Fig 1.5. At the N-terminal of the protein, there are two domains, which are reported to be responsible for the interaction with two other components of PRC2: (i) DomainI interacts with embryonic ectoderm development (EED) and (ii) DomainII interacts with Suppressor of Zeste 12 (SuZ12). EZH2 protein also consists of a nuclear localization signal (NLS) that allows it to transport into the nucleus. At the C-terminal, there are highly conserved cysteine-rich and SET domains, which were demonstrated to have methyltransferase activity (Cao and Zhang, 2004a). However, it was indicated that EZH2 has no enzymatic activity on its own (Cao and Zhang, 2004b; Muller *et al*., 2002). Only by forming a complex with EED and SuZ12, EZH2 could exhibit its methyltransferase activity. In fact, in the absence of EED or SuZ12, EZH2 protein stability was demonstrated to be disrupted. EZH1, a close relative of EZH2, was also being discovered in 1990s (Abel *et al*., 1996; Laible *et al*., 1997). However, only recently it was reported that EZH1 could also form PRC2 complex with EED and SuZ12 and exert methyltransferase activity albeit at a lower efficiency than EZH2 (Ho and Crabtree, 2008; Shen *et al*., 2008).

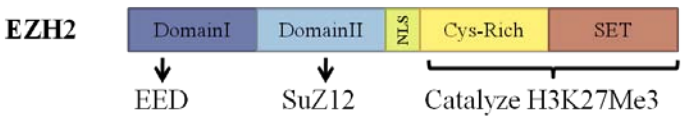

Figure 1.5 Protein Structure of EZH2.

EED contains WD40 repeat domain that was shown to have affinity binding to methylated histone 3 lysine 27 (H3K27), thereby aiding in the propagation of H3K27 trimethylation (H3K27me3) by PRC2 complex (Margueron *et al*., 2009). On the other hand, although SuZ12 was demonstrated to be essential for PRC2-mediated H3K27 trimethylation (Cao and Zhang, 2004b), the exact function of SuZ12 in the complex is unclear. Besides

EZH2, EED, and SuZ12 being the core complex of PRC2, several research groups had revealed additional subunits in PRC2, such as RBBP4/7, Jarid, and PCL, which help to enhance methyltransferase activity and efficiency, or the binding of the complex to histones (Figure 1.6).

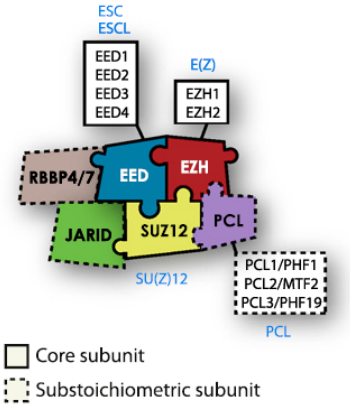

Figure 1.6 PRC2 complex formed by polycomb protein components. Modified from (Sauvageau and Sauvageau, 2010)

1.2.2 Modes of transcriptional repression

PRC2-catalyzed H3K27me3 near the promoter of its target genes is associated with transcriptional repression of these genes (Cao *et al*., 2002). There are several mechanisms proposed to be downstream of this histone methylation mark, which facilitate the silencing of the target gene expression. These include histone ubiquitination, DNA methylation, histone deacetylation, and chromosome remodeling.

1.2.2.1 Histone ubiquitination

As a consequence of trimethylation of H3K27 by PRC2 complex, another family of polycomb group (PcG) chromatin modifying complex, PRC1, would be recruited by the histone mark to catalyze monoubiquitination of histone H2A at lysine 119 (H2AK119Ub1) (Wang *et al*., 2004). Two RING-finger domain-containing proteins, RING1A and RING1B are the catalytic subunits of PRC1, in which the latter being the more predominant and efficient E3 ubiquitin ligase (Buchwald *et al*., 2006). B lymphoma Mo-MLV insertion region 1 (BMI1) is another core component of PRC1 complex that was reported to be essential for RING1B ubiquitin ligase activity (Cao *et al*., 2005). Importantly, PRC1 also contains chromobox homologues (CBX) proteins, which are crucial in binding to H3K27me3 mark. Studies have revealed that PRC1 mediated ubiquitination could lead to chromatin compaction (Francis *et al*., 2004) and impede transcriptional elongation (Stock *et al*., 2007), thereby leading to the repression of target gene expression.

1.2.2.2 DNA methylation

It was revealed that PRC2 complex could interact and thus recruit DNA methyl-transferases (DNMTs) to the target genes (Vire *et al*., 2006). DNMTs represent a family of proteins that catalyzes methylation on cytosine residues in the CpG islands (Robert *et al*., 2003). In mammalian cells, CpG islands are found mainly at the promoter region of open

reading frames. Upon methylation at CpG islands, the associated genes would be silenced by impeding direct binding or transcription factors to the promoter.

1.2.2.3 Histone deacetylation

In addition, PRC2 was also found to have direct physical interaction with HDACs and thus able to recruit HDACs to the target genes (Simon and Lange, 2008). Histone tails are positively charged due to high composition of Arginines and Lysines. On the other hand, DNA is highly negatively charged due to the presence of the phospho groups from phospho-diester backbone. As a consequence, when negatively charged DNA is wound around positively charged histones, the ionic bonding allows strong binding and causes the compaction of the chromatin, leading to higher level of the chromatin compaction and silencing of the genes.

1.2.2.4 Chromosome remodeling

Chromatin remodeler is a class of proteins that regulate nucleosome positioning and the subsequent transcriptional event. In cancers, SWI/SNF, a family of chromatin remodeling complexes, was frequently mutated or inactivated. This was found to be correlated with the suppressed expression of tumor suppressor genes like INK4A (Kia *et al*., 2008). It was found that the occupancy of PcG proteins could block the recruitment of SWI/SNF (Wilson and Roberts, 2011). The antagonism between SWI/SNF and EZH2 was further evidenced by another study demonstrating that the loss of SNF5 could lead to elevated expression of EZH2 and the *in vivo* tumorigenicity induced by SNF5 knockout could be rescued by EZH2 knockout (Wilson *et al*., 2010).

1.2.3 EZH2 and cancers

The oncogenic roles of EZH2 were first demonstrated in prostate cancer by a research group lead by Chinnaiyan in 2002 (Varambally *et al*., 2002). They found that EZH2 is overexpressed in prostate cancer and the overexpression is positively correlated with tumor stages. Higher expression of EZH2 in patient tumors confers worse prognosis. In the subsequent years, research on EZH2 intensified and EZH2 is now found to be overexpressed in multiple types of cancers including colon, bladder, lung, breast and prostate cancers (Figure 1.7).

In addition, ectopic overexpression of EZH2 in immortalized cell lines was demonstrated to be sufficient to promote neoplastic transformation (Kleer *et al*., 2003), indicating its role in early tumorigenic event. Further ectopic overexpression of EZH2 in weakly metastatic cancer cell lines was shown to enhance the motility and invasiveness of cancer cells (Collett *et al*., 2006; Varambally *et al*., 2002), implying that EZH2 also plays an essential role in promoting cancer progression to the advanced, metastatic stage.

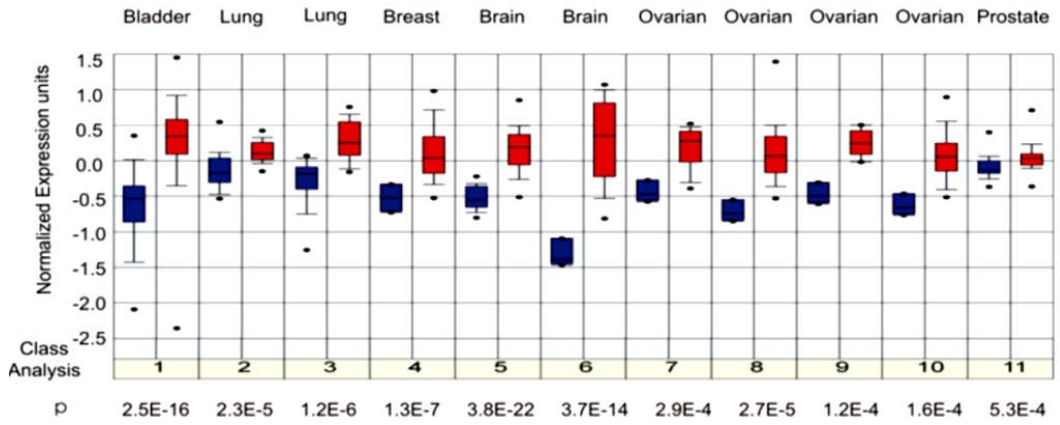

Figure 1.7 Overexpression of EZH2 in multiple cancer types. Blue bars, Normal tissue; Red bars, Tumor tissues. Adapted from (Yang *et al*., 2009a)

1.2.3.1 Transcriptional repression of tumor suppressor genes

The most well known mechanism behind EZH2-driven oncogenesis is via the repression of tumor suppressor gene expression by PRC2-dependent H3K27 trimethylation. The first evidence was reported in prostate cancer in which EZH2 was demonstrated to repress the expression of E-cadherin (Cao *et al*., 2008). EZH2-mediated silencing of E-cadherin causes EMT transition, thereby inducing cancer cell invasiveness. To date, EZH2 has been shown to repress multiple tumor suppressors that regulate different pathways in cancers (Fig 1.8). These target genes include BIM and FBXO32 that antagonize cell survival (Wu *et al*., 2011; Wu *et al*., 2010); DACT3 and DKK that suppress cell proliferation through modulating Wnt pathway (Hussain *et al*., 2009; Jiang *et al*., 2008); INK4A that induces senescence (Agherbi *et al*., 2009; Bracken *et al*., 2007); DAB2IP that inhibits invasion by repressing NF-κB and Ras pathways (Min *et al*., 2010); and VASH1 that suppresses angiogenesis (Lu *et al*., 2010).

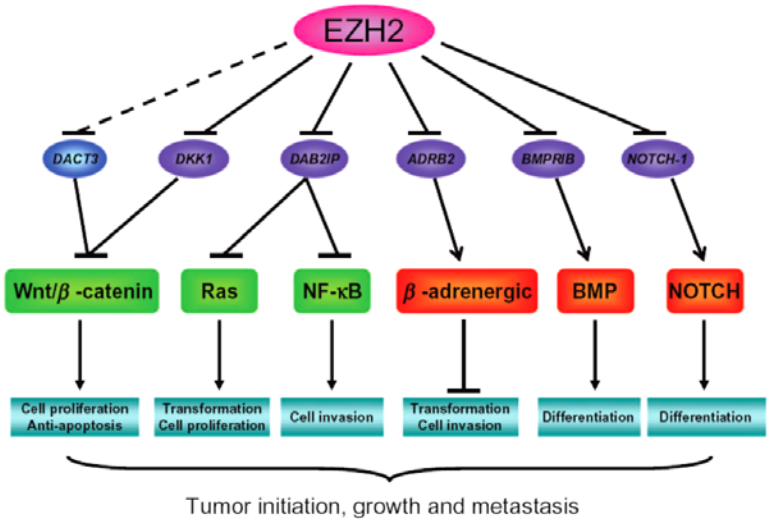

Figure 1.8 EZH2 mediates silencing of multiple genes involved in various oncogenic functions. Adapted from (Tsang and Cheng, 2011)

1.2.3.2 Histone methylation-independent functions

Besides transcriptional repression of tumor suppressors through H3K27me3, EZH2 was reported to have histone methylation independent functions which could lead to tumorigenesis. I-hsin Su *et al*. reported a cytosolic role of EZH2 complex, which involves the regulation of growth factor receptor-induced actin polymerization (Su *et al*., 2005). Such regulation requires the interaction between VAV1, a GTP/GDP exchange factor and EZH2/EED/SuZ12 complex in the cytoplasm. However, the function of EZH2 is still dependent on the methyltransferase activity of EZH2. As EZH2 could regulate actin polymerization, the authors implied that cancer cells in which EZH2 is overexpressed could have enhanced invasiveness arising from this regulation.

Another histone methylation independent function was reported by Shi Bin *et al.* They demonstrated that EZH2 has transactivating function in the nucleus by bridging the interaction between estrogen receptor and β-catenin (Shi *et al*., 2007). As the outcome, the transcriptional activity of these two transcription factors could be promoted, thereby enhancing the expression of the target genes like c-myc and cyclin D1, which would in turn enhance cancer cell proliferation. Interestingly, it was found that this function of EZH2 is independent of the methyltransferase function and does not require the engagement of other components of PRC2 complex.

1.2.4 Regulation of EZH2 in cancers

Currently, EZH2 is well-recognized as an oncogene in human cancers. Many papers have focused on how EZH2 is overexpressed, how its methyltransferase activity is regulated, and how EZH2 is directed to its targets in cancer cells.

1.2.4.1 Regulation of EZH2 expression

The mechanisms behind the overexpression of EZH2 in cancers could be dissected into three hierarchies: (i) transcriptional level; (ii) post-transcriptional mRNA level; and (iii) protein level.

At transcriptional level, EZH2 was found to be regulated by several transcription factors that are known to be overexpressed or overactivated in cancers. E2F1 is a very well-characterized transcription factor that exhibit dual roles in regulating cell survivability. It was revealed that E2F1 promote the transcriptional expression of EZH2 (Wu *et al*., 2010), which will inhibit E2F1-induced cell death by repressing Bim expression. Another group discovered that EZH2 is a direct transcriptional target of Elk1 under the regulation of Ras/MAPK/ERK pathway (Fujii *et al*., 2011). Besides, EZH2 expression level was revealed to be negatively regulated by p53 (Tang *et al*., 2004). More than 50% of human cancers harbor underexpression or mutation of p53, which in turn results in the release of transcriptional repression and subsequent elevation of EZH2 expression in these cancers.

The mRNA level of EZH2 could be further regulated by microRNAs (miRNAs), a subcategory of non-coding RNA that binds to 3'-untranslated region of mRNA to promote mRNA degradation by Dicer-associated machineries. *MiR-101*, a miRNA whose expression is lost during prostate cancer progression, was demonstrated to target *EZH2* mRNA for degradation (Varambally *et al*., 2008). Another report indicated that *EZH2* is also regulated

by *miR26a*, which the latter is suppressed by c-myc that is frequently overexpressed in cancers (Sander *et al*., 2008).

More recently, Zoabi *et al*. discovered that EZH2 and other components of PRC2 complex could be induced for protein degradation by a ubiquitin ligase, PRAJA1 (Zoabi *et al*., 2011). Interestingly, PRAJA1 itself is repressed by PRC2 complex and could be induced in cancer cells upon the treatment of EZH2 inhibitor, 3-Deazaneplanocin A (DZNep).

1.2.4.2 Regulation of EZH2 activity

The regulation of EZH2 methylation activity was mentioned in several studies. A research group lead by Mien-Chie Hung first discovered that EZH2 could be modified post-translationally by AKT through serine phosphorylation (Cha *et al*., 2005). In that study, the researchers noticed that the activated AKT could phosphorylate EZH2 at Serine 21, which would lead to the reduction of EZH2 binding to histone H3 and the subsequent H3K27 trimethylation. It was suggested that the released pool of EZH2 complexes would then be able to methylate other non-histone substrate that could drive tumor progression.

A few years later, the same group reported another phosphorylation site of EZH2 at Threonine 487 by cyclin-dependent kinase 1 (CDK1) (Wei *et al*., 2011). This phosphorylation causes the dissociation of EZH2 from PRC2 complex and thereby reducing H3K27me3 and the repression of the target genes. This regulation was shown to have negative effects on cancer cell migration and osteogenic differentiation of mesenchymal stem cells. On the contrary, almost at the same time, another group lead by Haojie Huang discovered that CDK1 and CDK2 could phosphorylate EZH2 at Threonine 350 (Chen *et al*., 2010). In contrast to phosphorylation at Threonine 487, the phosphorylation at Threonine 350 leads to increase DNA binding of EZH2 and the associated H3K27me3 mark and as a result, the invasive potential of the cancer cells increases.

In addition to phosphorylation, EZH2 was found to be sumoylated at multiple sites *in vitro* (Riising *et al*., 2008). However, the identity of the E3 ligase that is responsible for the sumoylation of EZH2 is still not known. Moreover, the occurrence and function of such modification in *in vivo* is still unclear. Further investigations need to be done to clarify these questions.

Besides post-translational modifications, it was discovered that mutations of EZH2 in cancer cells could impact its methylation activity. In B-cell lymphoma, EZH2 has a recurrent mutated site at Tyrosine 641 (Y641) that reside in the catalytic SET domain (Morin *et al*., 2010). This point mutation is found to be cancer-specific as it is only present in tumor DNA but not the normal counterpart. When Y641 is mutated, the methylation activity of EZH2 reduces drastically. Very often, B-cell lymphoma cells that harbor this mutation are normally heterozygous, in other words, only one out of the two homologous chromosomes harbors this mutation, while the other copy of EZH2 remains unmutated. It was later discovered that while wild type EZH2 exerts higher methylation activity on unmethylated H3K27 compared to mono- and di-methylated H3K27, EZH2 Y641 mutant shows higher activity on dimethylated H3K27 and almost no activity towards unmethylated or mono-methylated counterparts (Yap *et al*., 2011). Concordantly, when a tumor cell expresses of both wild type and Y641 mutant EZH2, H3K27 trimethylation activity will be enhanced as a result of the cooperation between the two variants. Another mutation of EZH2 at A677 was also discovered in lymphoma to synergize with Y641 mutant EZH2 in promoting hypermethylation of H3K27 (McCabe *et al*., 2012).

1.2.4.3 Steering of PRC2 binding to targets

Unlike polycomb complexes in Drosophila that have defined DNA binding motif, termed polycomb response element (PRE), polycomb complexes in mammals have less defined DNA-targeting signals (Cao *et al*., 2002). Although EED was found to have DNA binding affinity, the mechanisms behind specific recognition of PRC2 complexes to their

targets remains undetermined and has become a research focus in past decade. Thus far, two mechanisms for EZH2 recruitment to the chromatin were proposed: (i) recruitment by transcription factors and (ii) redirection by non-coding RNA (ncRNA).

Most transcription factors have distinct DNA sequences (or motif) that they would have exhibit higher affinity towards and preferentially bind. Upon binding to their respective DNA motifs, the transcription factors would then recruit transcriptional co-activators or co-repressors that facilitate the activation or repression of the target gene expression, respectively. EZH2-containing PRC2 complex is one such example of transcriptional co-repressor. Clara Hwang *et al.* demonstrated that EZH2 could physically bind to Repressor of Estrogen receptor Activity (REA) and suppress ER-mediated transcription (Hwang *et al*., 2008). Another study reported that IFNα-stimulation resulted in the recruitment of STAT2 and EZH2 complex to the promoter of *DNp73*, leading to its suppression (Testoni *et al*., 2011). A more recent study also demonstrated that upon activation of Androgen Receptor (AR), EZH2 and HDACs could be recruited together with AR co-repressor, ERG, resulting in genome-wide repression of AR target genes (Chng *et al*., 2012).

In addition to promoter recruitment of EZH2 complex by transcription factors, EZH2 binding to specific target genes could also directed by non-coding RNA. A long well-known example is the X-chromosome inactivation mediated by PRC2 recruitment to *Xist*, a non-coding RNA that is transcribed from the X-chromosome *in cis* (Plath *et al*., 2003). More recently, *HOTAIR*, a long non-coding RNA that is transcribed from HOXC locus on chromosome 12 and tend to bind to HOXD genes on chromosome 2 *in trans* to mediate HOXD gene repression by directly recruiting PRC2 complex (Kaneko *et al*., 2010; Rinn *et al*., 2007; Tsai *et al*., 2010).

1.2.5 EZH2 and breast cancer

EZH2 is known to be overexpressed in breast cancer and the overexpression level is correlated with breast cancer aggressiveness (Collett *et al*., 2006). Indeed, EZH2 is demonstrated to repress multiple tumor suppressor genes in breast cancer cells such as CDKN1C and RUNX3 (Fujii *et al*., 2008; Yang *et al*., 2009a). These genes are thought to be crucial at the early stage of tumorigenesis. In addition, EZH2 also suppresses CDH1 and FOXC1 (Cao *et al*., 2008; Du *et al*., 2012), two negative regulators of tumor invasion, which might be crucial in advance tumor progression. Based on the evidences in clinical samples, EZH2 was even proposed to be the prognostic marker of aggressive breast cancer.

More recently, evidences pointing to EZH2 being responsible for the expansion of breast tumor-initiating cells (also known as cancer stem cells) were emerging. It was shown that EZH2 represses the expression of RAD51 (Zeidler and Kleer, 2006; Zeidler *et al*., 2005), a protein that helps to repair DNA double-stranded break. With RAD51 suppressed, genomic instability ensues, culminating into the amplification of Raf1 locus. This increases the expression of Raf1 and leads to hyperactivation of ERK/β-Catenin pathway, which would in turn promote cell survival and proliferation of the breast tumor-initiating cells.

EZH2 overexpression is found to be more associated with ER-negative breast cancer, especially TNBC. Conversely, BRCA1 is often mutated and underexpressed in TNBC. Many studies have reported potential crosstalk between EZH2 and BRCA1 in TNBC (Gonzalez *et al*., 2011; Gonzalez *et al*., 2009; Kleer, 2009; Puppe *et al*., 2009). It was indicated that EZH2 represses BRCA1 function and the decrease in cell proliferation as a result of EZH2 inhibition requires the presence of BRCA1. It was further demonstrated that EZH2 promote nuclear export of BRCA1 to facilitate genomic instability. It was also shown that EZH2 inhibition by DZNep is more effective in suppressing growth of BRCA1-deficient breast cancer cells, highlighting the potential in targeting EZH2 in BLBC that is mostly BRCA1-deficient in nature.

1.3 NF-κB

1.3.1 NF-κB as a family of transcription factors

Nuclear Factor κB (NF-κB) represents a family of transcription factors that consisting of Rel-homology domain, which enables these transcription factors to form dimers and bind to DNA for transcription (Gilmore, 2006). There are five family members of NF-κB which could be categorized into two types (Figure 1.9): (i) with transactivational activity, includes RelA, RelB, and Rel; (ii) without transactivational activity, includes p50 and p52. These five family members could form dimers with different combinations to regulate the expression of specific target genes. In general, there are two pathways of NF-κB consisting of distinct stimuli, intermediate pathway mediators, and NF-κB components, respectively designated as canonical and non-canonical NF-κB pathways (Figure 1.10). In the past 25 years, NF-κB has been intensively studied as a result of its importance in immunology and cancer biology (Hayden and Ghosh, 2012). To date, about 5000 review papers are publically available. This has made a comprehensive review on all aspects of NF-κB unfeasible. Hence, in this thesis, I will only give a brief introduction of NF-κB pathways and focus primarily on the oncogenic roles of NF-κB in breast cancer.

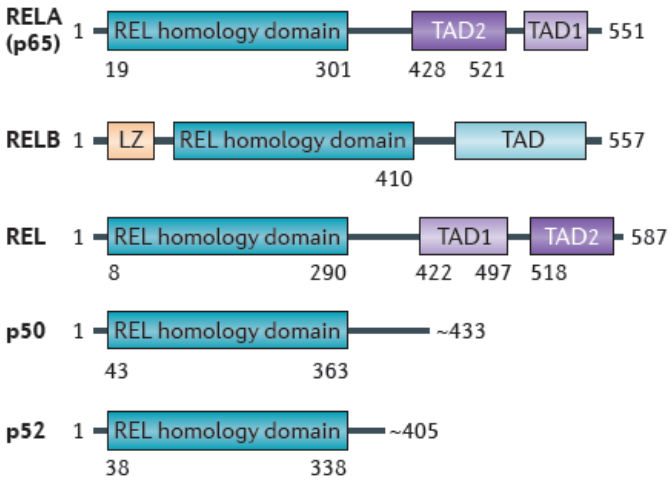

Figure 1.9 Protein structures of NF-κB subunits. Adapted from (Perkins, 2012)

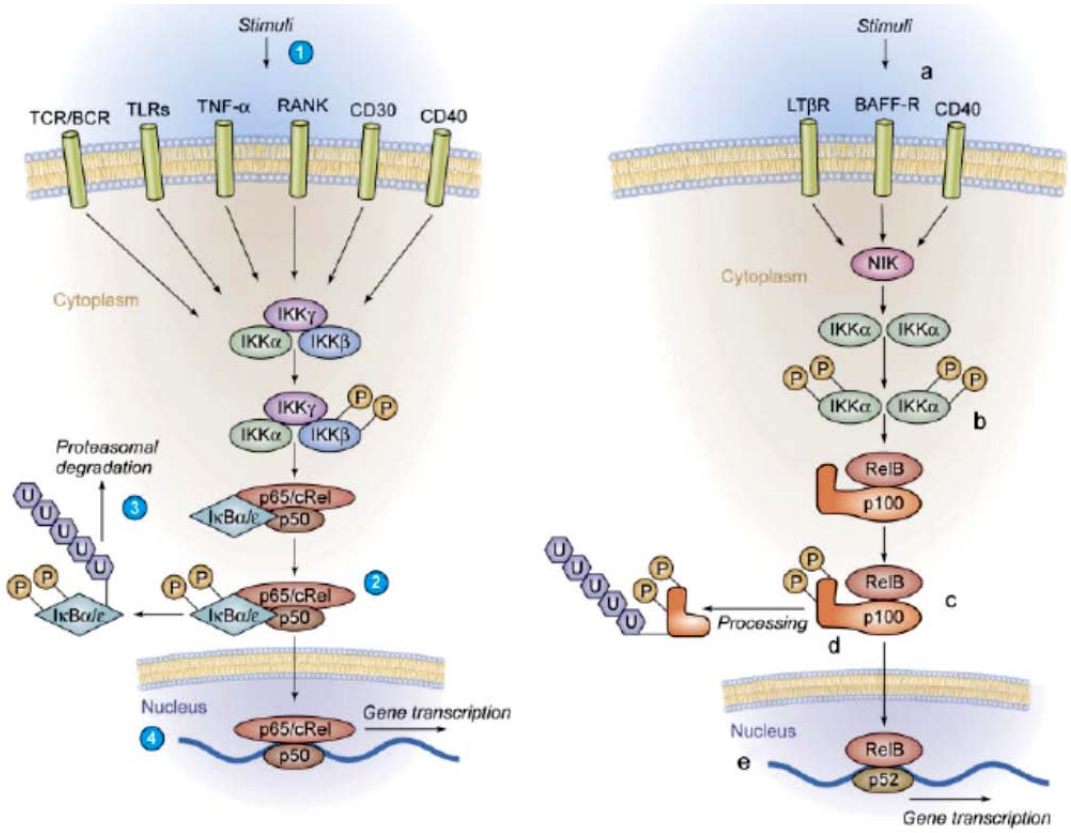

Figure 1.10 Canonical and non-canonical pathways of NF-κB. Adapted from (Jost and Ruland, 2007).

1.3.1.1 Canonical NF-κB pathway

Canonical NF-κB pathway is more widely studied as compared to the non-canonical NF-κB pathway. It was suggested that the canonical NF-κB pathway is defined by the involvement of NEMO (also known as IKKγ) in the activation of NF-κB transcription factors (Shih *et al.*, 2011). This pathway could be activated by a wide variety of stimuli including proinflammatory cytokines (e.g., TNFα and IL1), pathogen associated molecular patterns (PAMPs) (e.g., LPS), and cellular stress (e.g., DNA damage) (Pahl, 1999). NF-κB is normally expressed, dimerized, and sequestered by Inhibitor of κB (IκB) in the cytoplasm, poised for activation by appropriate stimuli. Upon stimulation, the IKK complex containing NEMO, IKKα, and IKKβ would be activated by phosphorylation. The phosphorylated IKKβ would in turn phosphorylate IκB, and lead to proteosomal-dependent degradation of the latter. In the canonical pathway, IκBα, the most common isoform of the IκB family, is predominantly

associated with RelA/p50 dimer prior to stimulation. After IκB is degraded, the transcription factors would then translocate into the nucleus to turn on specific gene transcription. In some studies, Rel was shown to have overlapping role as RelA, and is also implicated in canonical pathway (Gilmore, 2006).

A large cohort of target genes is regulated by RelA (Pahl, 1999), the transcriptional output of the target genes is fine-tuned by the type of stimuli, combinations of RelA dimer, and cellular context. In general, the canonical NF-κB pathway is recognized to play crucial roles in mounting innate immune response. Upon stimulation, a large number of genes would be upregulated, for instance cytokines (e.g., IL8, IL6, TNFα, and LTβ), adhesion molecules (e.g., ICAM1 and VCAM1), acute phase protein (e.g., SAA), and inducible enzymes (e.g., COX2). The elevation of these secretory molecules would recruit more inflammatory leukocytes to the site where the NF-κB pathway is stimulated.

Under normal condition, the activation of canonical NF-κB pathway is well-controlled temporally. After acute activation of RelA-containing dimer, several events would occur to quench and terminate NF-κB signaling. The negative feedback mechanisms include: (i) expression of IκB that could relocalize RelA-dimer back to the cytoplasm; (ii) expression of TNFAIP3 that could inhibit further activation of IKK; (iii) phosphorylation of RelA mediated by IKK in the nucleus could lead to the ubiquitination and degradation of RelA by E3 ligase PIAS1 (Shih *et al*., 2011).

1.3.1.2 Non-canonical NF-κB pathway

Non-canonical NF-κB pathway is demonstrated to be activated by a subset of TNF superfamily receptors (TNFSFRs), which includes lymphotoxin β receptor (LTβR), CD40 and B-cell activating factor receptor (BAFFR) (Razani *et al*., 2011). Upon stimulation, cellular inhibitor of apoptosis (cIAP1 and cIAP2) would be activated, causing the degradation of TRAF3 that could otherwise destabilize NF-κB-inducing kinase (NIK). As the result, NIK is

stabilized and able to phosphorylate and activate IKKα specifically. The activated IKKα would in turn phosphorylate and partially degrade p100 to produce p52, which preferentially binds to RelB. The intact p100 functions similarly as IκB to keep RelB in the cytoplasm by physical interaction through ankyrin repeats domain. After partial degradation, RelB/p52 dimer is released from sequestration and move to the nucleus to initiate gene transcription.

The target genes regulated under non-canonical NF-κB pathway are less-well studied. The physiological importance of these pathways was mainly demonstrated in mice with genetically modified NIK, IKKα, and p100 (Razani *et al*., 2011). It was shown that mice with disrupted non-canonical NF-κB signaling would manifest developmental defects of secondary immune organs like lymph nodes and spleen (Futterer *et al*., 1998), as well as B-cells development (Weih *et al*., 2001) and osteoclast differentiation (Vaira *et al*., 2008).

Unlike canonical NF-κB pathway, the activation of non-canonical NF-κB pathway is relatively slower but more sustained. However, little was known about the termination of this pathway. A recent study has shed light on this topic. It was found that the activated IKKα could feedback to phosphorylate and destabilize NIK, thereby regulating the activation of non-canonical NF-κB signaling (Razani *et al*., 2011).

1.3.2 NF-κB and cancers

NF-κB pathway has been implicated in multiple solid tumors as well as leukemia (Aggarwal *et al*., 2006; Jost and Ruland, 2007; Karin and Greten, 2005; Lee *et al*., 2009). NF-κB is reported to have ability to modulate the expression of tumor suppressor genes and oncogenes. It was thought that the silencing of key tumor suppressors like p53 and PTEN has skewed NF-κB activity to be pro-oncogenic (Mayo *et al*., 2002; Rocha *et al*., 2003). Up till now, the link of NF-κB to cancer has been well established. A couple of recent review articles have even suggested NF-κB-related inflammation as one of the hallmarks of cancer (Colotta *et al*., 2009; Hanahan and Weinberg, 2011).

1.3.2.1 Oncogenic functions of NF-κB

The oncogenic roles of NF-κB are mainly dependent on its ability to upregulate a large number of oncogenes involved in multiple aspects of oncogenesis (Jost and Ruland, 2007). The participation of NF-κB in oncogenesis is considerably extensive, from the basic features of tumor cells like enhanced cell proliferation, biological processes involved in advanced cancer progression like angiogenesis and metastasis, and even resistance to adjuvant therapy through promoting cancer cell survival.

Here, I provide an introduction on the series of events that describes how NF-κB could participate in the whole course of cancer progression (Perkins, 2012). Several cell cycle regulators are regulated by NF-κB such as Cyclin D1, Cyclin E, and CDK2 (Pahl, 1999). The upregulation of these genes enables cells to enter cell cycle progression and induces cell proliferation. Dysregulated expression of these genes would thus contribute to uncontrolled tumor growth. When tumor mass grows to a certain size, the oxygen supply to the tumor cells would become insufficient and enter a condition called hypoxia. As the result, NF-κB is activated by hypoxic elements released by the tumor cells (e.g., HIFα), angiogenic genes (e.g., VEGF) would then be turned on by NF-κB to induce blood vessels growth. The activated NF-

κB could also upregulate the expression of multiple pro-inflammatory cytokines such as TNFα, IL6, IL8, and IL1, which when released would attract tumor-infiltrating leukocytes. These leukocytes would further aggravate angiogenesis and promote cancer progression. The elevated expressions of adhesion molecules like ICAM1 and VCAM1 induced by NF-κB have been implicated in increased motility of cancer cells. In addition, NF-κB could also promote the expression of MMPs and uPA that helps to degrade extracellular matrix and facilitate invasion of cancer cells. Importantly, NF-κB regulates numerous pro-survival genes like BCL2, BCL-X_L, survivin, and BIRC3, which play critical roles in cancer cells survival especially in conferring chemoressistance in response to anti-cancer adjuvant therapy.

1.3.2.2 Constitutive activation of NF-κB in cancers

Under normal physiological conditions, NF-κB transcription factors are tightly regulated and sequestered in the cytoplasm. In cancers, however, NF-κB is observed to be constitutively active from the nuclear localization of the transcription factors observed in histoimmunochemistry staining of cancer tissue (Aggarwal *et al*., 2006; Lee *et al*., 2009). It is believed that constitutive activation of NF-κB cause persistent upregulation of pro-oncogenic target genes, leading to tumor progression.

Multiple mechanisms have been proposed to be accountable for constitutive activation of NF-κB in cancers. For instance, overexpression of upstream kinases like NIK and LTβR (Dhawan *et al*., 2008; Yamamoto *et al*., 2010) as well as the secretion of activating cytokines like TNFα and IL1β by tumor-associated leukocytes in tumor microenvironment would lead to continued NF-κB activation (Goldberg and Schwertfeger, 2010). Genetic DNA amplification of Rel in lymphoma is also proposed to be one of the causes of constitutive NF-κB activation (Barth *et al*., 2003). In additional, a shorten IκB half-life and mutations of IκB were also observed in B-cell lymphoma and Hodgkin lymphoma respectively (Sethi *et al*., 2008), would also release NF-κB from inhibition and lead to constitutively activation of NF-κB.

1.3.2.3 Targeting NF-κB in cancers

Due to the extensive involvement of NF-κB in cancer progression, this pathway served as an attractive therapeutic target (Aggarwal and Gehlot, 2009). To date, more than 700 inhibitors of NF-κB pathway have been identified (Gupta *et al*., 2010). These inhibitors encompass natural products, synthetic small molecules, and small DNA/RNA and peptides. NF-κB pathway can be targeted by these inhibitors at different unique components of the pathway (Gupta *et al*., 2010; Perkins, 2012; Prasad *et al*., 2010), for example: (i) IKK activity and IκB phosphorylation (e.g., sulindac and pomegranate extract); (ii) NF-κB nuclear translocation (e.g., SN50 and epicatechin); (iii) NF-κB post-translational modification (e.g., resveratrol and gallic acid); and NF-κB DNA binding (e.g., DHMEQ and sesquiterpene lactones).

Although enormous number of NF-κB inhibitors has been developed, not many of them were evaluated in terms of biological activity *in vivo* and cytotoxic effects. Among those inhibitors that were approved by US Food and Drug Administration (FDA), many of them are originally developed for other protein targets and in turn discovered to inhibit NF-κB pathway. These inhibitors include aspirin, which was intentionally used for inhibiting COX1/2 activity, but was later reported to inhibit IKKβ activity (Yin *et al*., 1998); simvastatin, which was initially used for controlling elevated cholesterol, was later shown to inhibit DNA binding of NF-κB (Lee *et al*., 2007). On the other hand, bortezomib, a proteasome inhibitor developed in attempt to inhibit NF-κB pathway by blocking IκB degradation, has obtained accelerated approval from FDA and was used for treatment of multiple myeloma (Kwak *et al*., 2011). However, it was found that bortezomib has a wider range of targets and the exact mechanism of its effectiveness is not well-defined (Yang *et al*., 2009b).

NF-κB is an essential player regulating normal physiological functions like immune responses and cellular homeostasis. Therefore, a major challenge for the development of NF-κB pathway inhibitors in cancer is the lack of specificity in targeting only the oncogenic

activity of NF-κB in cancer cells. Nevertheless, the use of aspirin (anti-inflammatory agent) in preventing colorectal cancer in high risk individuals has demonstrated the potential of targeting NF-κB in cancer treatment (Cuzick *et al*., 2009).

1.3.3 NF-κB in breast cancer

Similar to other cancers, NF-κB was also found to be constitutively active in breast cancer (Bhat-Nakshatri *et al*., 2002; Nakshatri *et al*., 1997). However, the mechanism underlying constitutive activation of NF-κB is not well elucidated. Two independent studies have indicated that transient activation of SRC or MEK/ERK pathway could initiate NF-κB activation and the subsequent feed forward signaling for self-sustainable NF-κB constitutive activity (Iliopoulos *et al*., 2009; Rokavec *et al*., 2012). Noteworthy, IL6 was implicated to have a crucial role in the feed forward maintenance of NF-κB signaling in breast cancer.

More than a decade ago, NF-κB was implicated to play a role in driving breast cancer progression (Nakshatri *et al*., 1997). It was found that when breast cancer progresses from a hormonal-dependent, non-metastatic form to higher grade hormonal-independent and metastatic form, the activity of NF-κB would be elevated. In addition, constitutive activation of NF-κB is reported to occur more frequently in basal-like breast cancer (BLBC) cells, which are mostly ER-negative (Gionet *et al*., 2009). Indeed, a recent study showed that ectopic expression of ER in ER-negative breast cancer cells could suppress the expression of RelB (Wang *et al*., 2007). On the other hand, RelB was also found to be able to suppress ER expression (Wang *et al*., 2009). These findings highlight the antagonistic roles of ER and NF-κB, particularly on the non-canonical pathway.

Consistent with the findings showing constitutive activation of NF-κB in advanced metastatic breast cancer, multiple target genes of NF-κB were also reported to enhance the invasiveness and metastatic potential of breast cancer cells. For instance, IL8 was shown to increase breast cancer cells *in vitro* and *in vivo* (Wu *et al*., 2008) and is elevated in serum of patients experiencing breast cancer cell dissemination (Benoy *et al*., 2004). Another NF-κB target, TNFα, was demonstrated to increase cell invasion when applied to breast cancer cell lines (Cho *et al*., 2009; Kim *et al*., 2008). Last but not least, IL6 was revealed to induce breast cancer cells motility and was proposed as a negative prognostic marker of metastasis

(Knupfer and Preiss, 2007). Furthermore, IL6 and IL8 was implicated in the maintenance of breast cancer stem cells (Iliopoulos *et al*., 2009; Iliopoulos *et al*., 2011; Liu and Wicha, 2010; Schafer and Brugge, 2007). IL6 (Conze *et al*., 2001) and BIRC3 (Hernandez-Vargas *et al*., 2007) were also reported to confer resistance to chemotherapeutic agents.

1.4 Aims and Objectives of Study

Basal-like breast cancer (BLBC) cells are often triple-negative, which represents a subset of breast cancer that lacks the expression or cell surface receptors, ER, PR, and HER2. This property has rendered BLBC irresponsive to receptor-targeted therapy. Furthermore, the poor prognosis of BLBC patients has made it a focus of research in order to develop a better approach in treating this subtype of breast cancer. The aim of this study is to dissect the role of EZH2 in BLBC, due to its elevated expression in this breast cancer subtype. By using small-interfering RNA to deplete EZH2 in BLBC model cell lines, we identified the crosstalk between EZH2 and NF-κB pathway in BLBC. Similar to the case of EZH2, NF-κB was found to be constitutively active in BLBC. Therefore, we sought to delineate the underlying mechanism of EZH2 regulation on NF-κB activity in BLBC as well as the clinical relevance of such regulation.

In addition, we were interested to study the function of EZH2in luminal breast cancer, which harbors high expression of EZH2 aibeit a lower level than BLBC. On the contrary, luminal breast cancer was known to have low activity of NF-κB signaling. Hence, we aimed to further understand the potential crosstalk between EZH2 and NF-κB in luminal breast cancer.

Taken together, different functions of EZH2 would be dissected in relation to the regulation of NF-κB pathway under two different cell contexts, namely BLBC and luminal breast cancer.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture and treatments

MDA-MB-231, BT549, MCF7, and T47D cell lines were obtained from American Type Culture Collection (Manassas, VA). The normal breast epithelial cell line, HMEC, was a generous gift from Dr. W.C. Hahn of Dana-Farber Cancer Institute. MDA-MB-231, BT549, MCF7, and T47D cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5000U/mL penicillin/streptomycin (Invitrogen). HMEC normal breast epithelial cell line was grown in DMEM/F12 supplemented with 5% horse serum, 20ng/ml EGF, 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10μg/ml insulin, and penicillin/streptomycin (Invitrogen). All cells were maintained in logarithmic monolayer growth in $75cm^2$ flask at 37°C with 5% CO_2 in a humidified atmosphere. For ChIP and co-immunoprecipitation assays, cells were treated with 10ng/ml TNFα (Calbiochem) for 2 hours prior harvest. For quantitative RT-PCR assays and Microarray gene expression analyses, cells were treated with 10ng/ml TNFα for 4 hours prior harvest.

2.2 Cryopreservation of cell lines

All cell lines were grown and maintained for less than 30 times passages. After which, frozen cells with lower passage number from liquid nitrogen tank (-196°C) were thawed at 37°C and neutralized with 6ml complete media. Cells were spun down at 800rpm for 5 minutes and cell pellets were resuspended with fresh complete media and seeded into T25 flasks. Cells were allowed to stabilize for 1 week before experiments were conducted. To freeze down cells for cryogenic preservation, cells were trypsinized and spun down at 100rpm for 3 minutes. Cell pellets were then resuspended with freezing media (For HMECs: 90% culture media and 10% DMSO; for other cell lines: 90% FBS and 10% DMSO) and aliquoted 1ml of $1x10^6$ cells into each cryovials. Two-step frozen process were adopted, cells were first placed in Mr. Frosty

cylinder (NALGENE) filled with 99% iso-propanol and kept at 80°C freezer. After 24 hours of incubation, cells were then transferred to liquid nitrogen tank.

2.3 Transfection of Small interfering RNA

siRNA and plasmids transfections were conducted using Lipofectamine RNAiMax (Invitrogen) and FugeneHD (Roche Applied Science), respectively according to the manufacturer's instructions. For siRNA transfection, 5ul of siRNA (20uM) and 4ul of RNAiMax were separately diluted into 100ul basic media. After 5 minutes of incubation, the two diluents were mixed together and incubate for another 20 minutes before adding the transfection mixture into 800ul complete medium-containing 6 well-plate, which was seeded with monolayer cells 18 hours prior transfection. The working concentration of siRNA was 100nM. The transfected cells were either being trypsinized 24 hours post transfection and reseeded for downstream assays or changed media 24 hours post transfection and further incubated until the time of harvesting. For transient overexpression plasmid transfection, Target-specific siRNA and non-targeting control siRNA were purchased from 1st Base Singapore with the following target sequences: EZH2 siRNA: 5'-GACUCUGAAUGCAGUUGCU-3'. EZH2 siRNA 5'-UTR; 5'-CGGUGGGACUCAGAAGGCA-3'; RelA siRNA: 5'-GCCCUAUCCCUUUACGUCA-3'; RelB siRNA: 5'-GCCCGUCUAUGACAAGAAA-3'; ERα. siRNA: 5'-UCAUCGCAUUCCUUGCAAA-3'.

2.4 Transfections of transient overexpression plasmids

pcDNA3-RelA and RelB were purchased from Addgene. pcDNA4-EZH2 wild type and SET domain deletion plasmid have been described previously (Wu *et al*., 2011). To perform transient overexpression for exogeneous co-immunoprecipitation, 5ug of pcDNA3-RelA, 5ug of pcDNA3-RelB, and 5ug of pcDNA4/pcDNA4-EZH2/pcDNA-EZH2-SETΔ were diluted in 500ul basic medium and incubate for 5 minutes. 45ul of FugeneHD was then added (1ug plasmid: 3ul FugeneHD ratio) followed by 20 minutes of incubation. The transfection mixture

was then added into 4.5ml complete medium-containing 10cm petridish, which was seeded with monolayer cells 18 hours prior transfection. The media of the transfected cells was changed with fresh complete DMEM 24 hours post transfection and samples were harvested 72 hours post transfection.

2.5 Generation of stable overexpression cell lines

Target genes from their respective transient expression plasmids were amplified by PCR and subcloned into the pMN GFP/IRES retroviral expression vector (obtained from Dr. Linda Penn's lab). To generate stable overexpression cell lines, 4ug of plasmids and 10ul of Lipofectamine 2000 were separately diluted into 100ul basic media. After 5 minutes of incubation, the two diluents were mixed together and incubate for another 20 minutes before adding the transfection mixture into 800ul OptiMEM-containing 6 well-plate (collagen I coated, BIOCOAT from BD), which was seeded with monolayer platinum-A retroviral packaging cells (CELL BIOLAB, INC., San Diego, CA) 18 hours prior transfection. After 6 hours of incubation, transfection media were replaced by 2.5ml fresh complete DMEM. After 48 hours post transfection, conditioned media from the transfected PlatA cells, which now contained retroviruses packaged with our target genes, were harvested and filtered through 0.45um filter unit. Destination cells were seeded in 6-well plate 24 hours prior viral infection. 2ml conditioned media were then added to 1ml complete media containing 3ul of 8ug/ml polybrene in the destination cells, followed by centrifugation at 1800rpm for 90 minutes. 48 hours post viral infection, destination cells were trypsinized and grew in culturing flasks. Viral infected cells were sorted based on GFP over-expression.

2.6 RNA extraction

Cell pellets were collected by harvesting monolayer cells by trypsinization. 1ml Trizol (Invitrogen) was added to lyse the cell pellet, followed by the addition of 200ul chloroform and centrifugation at 12krcf for 15 minutes at 4ºC to separate RNA, DNA, and proteins from

the cell contents. After centrifugation, the top liquid phase containing RNA was transferred to fresh eppendorf tube followed by addition of 70% ethanol. To purify RNA, RNeasy mini kit (Qiagen) was used. Briefly, the samples were transferred to the column to allow RNA binding and flow through were accelerated by centrifugation at 10krpm for 30 seconds. Columns were washed once with 750ul RW1 buffer and twice with 500ul RPE buffer. Finally, RNA was eluted with 40ul RNAse-free water. RNA concentration and purity were assessed using NAnodrop ND-1000.

2.7 cDNA conversion and Quantitative real-time PCR (RT-PCR)

RNA samples were reverse transcribed and converted to single-stranded complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). In brief, 750ng of RNA samples were diluted in 25ul nuclease-free water and added with 25ul of master mix containing 5ul RT buffer, 5ul random primers, 2ul dNTP mix, and 2.5ul MultiScribe^{TM} reverse transcriptase and 10.5ul nuclease-free water. The reaction mix was then subjected to PCR in thermo cycler running for 10 minutes at 25ºC followed by 2 hours at 37ºC. To perform quantitative real-time PCR, 0.44ul of cDNA samples (15ng/ul) were added together with 0.4ul gene-specific primer mix (10uM) to 5ul of 2X master mix from KAPA SYBR® FAST qPCR Kits (Kapa Biosystems) and top-up to 10ul with nuclease-free water. Finally, the reaction mix was amplified and quantified with PRISM 7900 Sequence Detection System (Applied Biosystems).

2.8 Microarray Gene Expression Profiling and Analyses

The microarray hybridization was performed in using the Illumina Gene Expression Sentrix BeadChip HumanRef-8_V2 (San Diego, CA) following manufacturer's protocol. Briefly, 500ng RNA samples were reverse transcribed into cDNA and further processed into double-stranded cDNA. After purification of cDNA, biotinylated cRNA was generated and further purified. The biotiylated cRNA was hybridized onto BeadChip and stained with streptavidin-Cy3 after washing. Finally, BeadChip was scanned using Illumina BeadArray Reader and

images were stored with barcodes indicated. The scanned images were processed using Illumina GenomeStudio™ and the generated data were imported into GeneSpringGX™ (Agilent Technologies) for further analysis. Gene expression data were deposited at NCBI Gene Expression Omnibus (GEO) repository database under the accession number GSE30670. Using GeneSpringGX, the data were analyzed by selecting Illumina single color as experimental type and signals were normalized to median expression. Fold changes were analyzed by pairwise comparisons to appropriate controls. To generate heatmaps, median-normalized data represented in $\log_2$ values were processed by Cluster and visualized by Treeview softwares (Eisen, 1998).

2.9 Gene Ontology analysis

Genes that were expressed with two-fold differences after EZH2 depletion in MB231 were separated into upregulated and downregulated genesets. The two genesets were then imported into Ingenuity Pathway Analyses (IPA) software for gene ontology analysis. From the analysis, signaling pathways and biological functions enriched in the imported genesets were obtained. Genes that appeared in the analyzed geneset and enriched in the biological function was indicated in the signalling network exported from IPA.

2.10 Protein extraction

Cell pellets were collected by harvesting monolayer cells through trypsinization. For total protein extraction, cell pellets were then resuspended with 30ul to 40ul radioimmunoprecipitation assay lysis buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 150mM NaCl, 1% Igepal CA630, 0.5% sodium deoxycholate, 1mM Na_2VO_4, 20mM NaF, 1mM PMSF, and Complete protease inhibitor (Roche)). Samples were incubated on ice for 30 minutes, vortexed for 15 seconds every 5 minutes. Subsequently, samples were sonicated twice for 5 seconds followed by centrifugation at 13.2krpm for 15 minutes at 4ºC. Supernatant was transferred to fresh eppendorf tubes and protein concentration was estimated with DC Protein Assay (Bio-Rad) using BSA with known concentrations as the standard and

measured using Tecan Xfluor™ software. Nuclear and cytoplasmic fractionation was conducted using NE-PER Fractionation Kit (Pierce) following manufacturer's protocol.

2.11 Western Blotting

Protein samples (20-30ug) were separated by 8% or 10% SDS-PAGE gel and subsequently transferred onto PVDF membrane (Millipore) using Trans-Blot SD Semi-Dry transfer cell (Bio-Rad). Membranes with immobilized proteins were blocked with 5% non-fat milk (Bio-Rad) for 30 minutes followed by primary antibodies for 1 hour and HRP-conjugated secondary antibodies for 45 minutes. Membranes were incubated with chemoluminescent ECL Substrate Kit (Amersham) and signals were detected using Kodak films. Anti-EZH2 (CS-3147) and ERα (CS-2512) was purchased from Cell Signaling. Anti-RelA (sc-8008) and anti-RelB (sc-48366) were obtained from Santa Cruz, anti-SuZ12 (39357) was obtained from Active Motif, anti-trimethylated H3K27 (07–449), anti-H3 (06–599), were purchased from Upstate. Anti-Myc and anti-actin were purchased from Roche Applied Science and Sigma-Aldrich, respectively.

2.12 Co-immunoprecipitation (co-IP)

50ug nuclear protein lysates were diluted with 500ul IP lysis buffer and precleared with 30ul Protein A Sepharose beads (Roche) for 4 hours. Samples were centrifuged at 12krcf for 20 seconds and supernatants were transferred to fresh eppendorf tubes. 2ug of specific primary antibodies were added and samples were rotated overnight at 4ºC. 25ul Protein A beads were added the next day morning and further incubated for 1 hour. Beads that now contained immunoprecipitated samples were washed once with IP Lysis buffer, once with Washing buffer 2 and once with Washing buffer 3. Immunoprecipitates were eluted with SDS loading buffer and analysed with Western Blotting. For endogeneous co-IP, less stringent lysis/washing buffers were used: IP Lysis buffer (Triton X-100: 1%; NaCl: 150 mM; 20 mM Tris-HCl pH8.0); Washing Buffer 2/3 (50mM Tris-HCl pH7.4; NaCl 150mM); for exogeneous co-IP, more stringent lysis/washing buffers were used: IP Lysis buffer (50mM

Tris-HCl pH7.4, 150mM NaCl, 0.5% sodium deoxycholate, 0.5% Igepal CA630); Washing buffer 2 (50mM Tris-HCl pH7.4, 500mM NaCl, 0.05% sodium deoxycholate, 0.1% Igepal CA630); Washing buffer 3 (50mM Tris-HCl pH7.4, 0.05% sodium deoxycholate, 0.1% Igepal CA630). Anti-EZH2 (#39901, Active Motif), anti-RelA (sc-109), anti-RelB (sc-226), anti-ERα (SC-543) or a non-specific IgG (sc-2027) was used in the co-IP assay. Densitometry was performed using UN-SCAN IT gel™ V6.1 software (Silk Scientific), following manufacturer's instruction.

2.13 Chromatin Immunoprecipitation (ChIP) and Sequential ChIP

Cells were expanded in 15cm petridish. To harvest for ChIP assay, cells were fixed with 3.7% paraformaldehyde for 20 minutes with gentle shaking followed by neutralization with 2ml 2M glycine for 5 minutes with gentle shaking. Fixed cells were washed twice with 10ml cold PBS and subsequently harvested by scrapping. Cell pellets were then lysed with 500ul SDS lysis buffer (1%SDS, 5mM EDTA, 50mM TrisHCl pH8) and sonicated for 6.5 minutes (30 seconds pulse on and 30 seconds pulse off) followed by centrifugation at 13.2krpm for 10 minutes. Supernatants were then transferred to 15ml Falcon tubes and top-up with Dilution buffer (1% TritonX100, 2mM EDTA, 20mM TrisHCl pH8, 150mM NaCl) to 3ml. Samples were then precleared with 210ul BSA blocked Protein A beads (Zymed) and 1ug normal IgG for 4 hours with rotation at 4ºC. Pre-cleared chromatin was immunoprecipitated with 10ug specific primary antibodies and rotated overnight at 4ºC. 80ul Protein A beads were added the next day morning and further incubated for 1 hour. Beads were then washed ten minutes each, once with 5ml TSEI buffer (0.1% NP40, 1% TritonX100, 2mM EDTA, 20mM TrisHCl pH8, 150mM NaCl), once with 5ml TSEII (0.1% SDS, 1% TritonX100, 2mM EDTA, 20mM TrisHCl pH8, 500mM NaCl), once with 5ml Buffer III (0.25M LiCl, 1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10mM TrisHCl pH8), and once with 5ml TE Buffer (2mM EDTA, 10mM TrisHCl pH8). Immunoprecipitated chromatins were eluted with 240ul SDS Elution Buffer (1%SDS, 10mM EDTA, 50mM TrisHCl pH8) and shaked at 65ºC for 30 minutes. Supernatants were transferred to fresh eppendorf tubes and decrosslinked overnight

at 65ºC. Purification of immunoprecipitated DNA was performed using PCR purification kit (Qiagen) following manufacturer's protocol. The immunoprecipitated DNA was quantitated by real-time quantitative PCR using KAPA SyBr Fast qPCR kit (KAPA Biosystems). The enrichment of specific genomic regions was assessed relative to the input DNA followed by normalization to the respective control IgG values. Primer sequences were listed in the table below. Anti-EZH2 (#39901, Active Motif), anti-RelA (sc-109), anti-RelB (sc-226), Anti-H3K27me3 (Upstate 07-449), anti-ERα (SC-543) or a non-specific rabbit IgG (sc-2027) was used in the ChIP assay. Primer sequences were listed in the table below.

Sequential ChIP was performed with similar procedures as ChIP assay except that first ChIP antibodies were crosslinked to Protein A beads with disuccinimidyl suberate (DSS) (Pierce) prior to the first immunoprecipitation process. Immunoprecipitated chromatin from the first IP was eluted with SDS elution buffer at 37ºC for 45 minutes. The eluate was then subjected to second IP using the same protocol as mentioned above.

2.14 Recombinant Protein Expression

RelA and RelB genes were subcloned into pDEST-HisMBP vector and EZH2 gene was subcloned into pDEST565 vector. The plasmids were then transformed into BL21 bacteria strain and the protein expression was induced by 0.4uM IPTG for 16 hours at 23C. Bacteria cultures were spun down 9krpm for 7 minutes, and resuspended with 30ml Lysis buffer (MBP lysis/washing buffer: 50mM Tris-HCl pH8, 0.1% β-ME, 100mM NaCl, 0.1% Triton-X100, protease inhibitors; GST Lysis buffer: PBS added with protease inhibitors). PBS complemented with Complete™ protease inhibitors. After a cycle of freeze-thaw, bacteria lysates were sonicated for 30 minutes (3 seconds pulse on, 2 seconds pulse off). Protein lysates were obtained by centrifugation at 20krpm for 1 hour at 4˚C. The MBP-tagged recombinant proteins were then purified using amylose resin (Biolabs) and eluted with MBP Elution buffer (50mM Tris-HCl pH8, 0.1% β-ME, 100mM NaCl, 0.1% Triton-X100, 10mM maltose, protease inhibitors); whereas the GST-tagged recombinant proteins were purified using glutathione sepharose beads (Amersham) and eluted with PBS Elution buffer (50mM

Tris-HCl, pH8.0, 150mM NaCl, 10mM reduced glutathione, 10mM DTT, 0.1% Triton X100, and protease inhibitors). Protein concentrations were determined using DC Protein Assay (Bio-Rad).

2.15 *In vitro* pull down and re-IP

50ug of MBP or MBP-tagged RelA or RelB was respectively incubated with 50ug GST-tagged EZH2 and 20ul of amylose resin. The pulls down reactions were incubated for 16 hours at 4˚C. The beads were then washed with MBP lysis/washing buffer (50mM Tris-HCl pH8, 0.1% β-ME, 100mM NaCl, 0.1% Triton-X100) and finally eluted using SDS lysis buffer and (Cell Signaling) followed by Western Blot analysis. For re-immunoprecipitation, MBP-tagged RelA and RelB were treated with TEV protease for 1 hour at room temperature. Next, 12ug RelA and RelB were incubated for 16 hours at 4˚C with 5ug GST or GST-EZH2, which was bound to 20ul glutathione sepharose beads. The beads were then washed as described in co-IP assay. Pulled down complexes were eluted with GST elution buffer. The eluted protein complexes were then subjected to co-IP assay as described earlier.

2.16 Transwell Invasion Assay

24-well FluorBlok transwell inserts (#351152, BD Biosciences) with a pore size of 8um were pre-coated with 80ul of growth factor-reduced matrigel (500ug/ml) (#356231, BD Biosciences) for 5 hours at 37˚C. $5x10^4$ MDA-MB-231 depleted of EZH2, RelA, and/or RelB were seeded in each insert with 200ul DMEM containing 0.5% FBS. 750ul DMEM supplemented with 0.5% FBS and 100ng/ml EGF was added outside the chamber as chemoattractant. Invaded cells were fixed after 48 hours of incubation by using 3.7% formaldehyde and stained with 25ug/ml propidium iodide (Sigma). 10 fields per inserts were scanned and numbers of invaded cells were counted with Cellomics ArrayScan.

2.17 3D Matrigel Anchorage-Independent Growth Assay

8-well chamber slides (#384118, BD Biosciences) were pre-coated with 45ul of 7.6mg/ml growth factor-reduced matrigel (#356231, BD Biosciences) for 30 minutes at 37℃. $5x10^3$ MDA-MB-231 depleted with EZH2, RelA, and/or RelB were seeded in each well with 400ul DMEM containing 10% FBS and 150ug/ml matrigel. Media was replaced with fresh media before phase contrast images were captured for 8 days at 4 days interval.

2.18 Dual Luciferase Reporter Assay

NF-κB-specific reporter plasmid pGL4.32, its negative control pGL4.15, and pRL-null were purchased from Promega. During transfection, 500ng pGL4/pGL4-NF-κB and 20ng pRL-null were diluted in 50ul basic media. After 5 minutes of incubation, 2.6ul FugeneHD was added and further incubated for 20 minutes. The transfection mixtures were then added into 400ul complete medium-containing 24-well plate, which was seeded with monolayer cells 18 hours prior transfection. The media of the transfected cells was changed with fresh complete media 24 hours post transfection. Cells were harvested 48 hours post transfection and luciferase activity was detected using the Dual Luciferase system (Promega) following manufacturer's protocol. For rescue assay, similar transfection method was adopted except Lipofectamine 2000 was used as the transfection reagent to transfect plasmids and siRNA simultaneously. To analyze luciferase activity, Firefly signals of pGL4/pGL4-NF-κB were normalized to Renilla signals of pRL-null in respective samples. pGL4-NF-κB/pRL-null ratio were further normalized to pGL4/pRL-null ratio to obtain normalized values corrected for the changes of basic transcription activity for indicated treatment of the cells.

2.19 Clinical Datasets and Survival Analysis

The expression data for the 54 breast cancer cell lines was described previously (Neve *et al*., 2006). The breast cancer data set from Farmer and Netherlands cohort with relevant clinical information has been described previously (Bos *et al*., 2009; Farmer *et al*., 2005). The EZH2/RelA/RelB co-regulated gene subset was used to classify breast cancer patients into higher expression or lower expression groups based on the mean of the expression of the subset of genes. Using the survival event status and time information, we computed the survival association of expression status (high/low expression) using Cox-Proportional Hazards model implementation (coxph) available in R-library "survival". Kaplan-Meier survival analysis was used for the analysis of clinical outcome.

2.20 Statistical Analysis

Statistical analyses of the bar graphs in this study were performed using Prism (GraphPad software Inc.). Student t-tests were carried out on triplicated experimental readings to generate p-values for pairwise comparisons.

CHAPTER 3: EZH2 AND NF-KB CROSSTALK IN BASAL-LIKE BREAST CANCER

3.1 EZH2 Positively Regulates NF-κB-Mediated Gene Network in Aggressive BLBC Cells

To gain mechanistic insights into the role of EZH2 in aggressive breast cancers, we sought to determine EZH2-dependent gene expression in basal-like breast cancer cells. To this end, we depleted EZH2 by small interference RNA (siRNA) in a widely used BLBC cell line, MDA-MB-231 (hereafter named MB231) cells and performed gene expression profiling using Illumina 24K human BeadArray-V2. Gene expression analysis led to identification of 836 genes that were differentially expressed upon EZH2 depletion (using two-fold cut-off, $p<0.01$), including 391 genes up-regulated and 445 genes down-regulated, respectively (Table S1). The great number of downregulated genes indicates with interest that in addition to the well-known function of EZH2 in gene repression, EZH2 may also have a similar capacity in promoting gene activation, even though it could be an indirect effect. To gain further insights into the biological functions of EZH2, we performed Ingenuity Pathway Analysis (IPA) with the two separate groups of genes and identified several gene networks that were highly enriched in EZH2 regulated genesets (Figure 3.1). Most networks identified were correlated with the known transcriptional repression functions of EZH2 such as cell cycle, cell death, cellular movement, and developments. Interestingly, inflammatory response was one network that was highly enriched only in EZH2 knockdown downregulated genesets (Figure 3.1B).

From the introduction, we know that inflammatory responses are mainly mediated by NF-κB transcription factors. As expected, the illustrated inflammatory network exported from IPA displayed a well interconnected regulatory system with NF-κB as a central node (Figure 3.2A). This suggested that NF-κB might be the key modulator of the inflammatory response under the regulation of EZH2. 36 genes enriched in the inflammatory network were further illustrated in a heatmap representing their expression profile after EZH2 depletion in MB231 (Figure 3.2B). From the heatmap, we noticed that many well-known NF-κB target genes such as *IL6*, *IL8*, and *PTGS2* being down-regulated for more than four-fold ($p < 0.05$, derived from

the triplicate microarray results). This observation raised a possibility that EZH2 may positively regulate NF-κB-mediated inflammatory gene network in MB231 cells.

A EZH2 knockdown upregulated geneset

Top network functions	Score
Infection Mechanism, Cardiovascular System Development and function, Organismal Development	42
Cell Death, Cell Signaling, Cellular Assembly and Organization	31
Cellular Movement, Dermatological Diseases and Conditions, Genetic Disorder	30
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	29
Cardiovascular Disease, Cardiovascular System Development and Function, Organismal Development	28

B EZH2 knockdown downregulated geneset

Top network functions	Score
Cell Cycle, Cancer, Genetic Disorder	38
Cell Death, Cellular Growth and Proliferation, Cell Cycle	36
Inflammatory Response, Cellular movement, Hematopoiesis	31
Organismal Injury and Abnormalities, Cancer, Gastrointestinal Disease	31
Cell Cycle, Connective Tissue Development and Function, Organismal Injury and Abnormalities	31

Figure 3.1 Ingenuity pathway analyses of genesets regulated by EZH2 in MB231

A. Ingenuity Pathways Analysis (IPA) showing the top gene networks enriched in upregulated genes following EZH2 depletion in MB231 cells.
B. Ingenuity Pathways Analysis (IPA) showing the top gene networks enriched in upregulated genes following EZH2 depletion in MB231 cells.

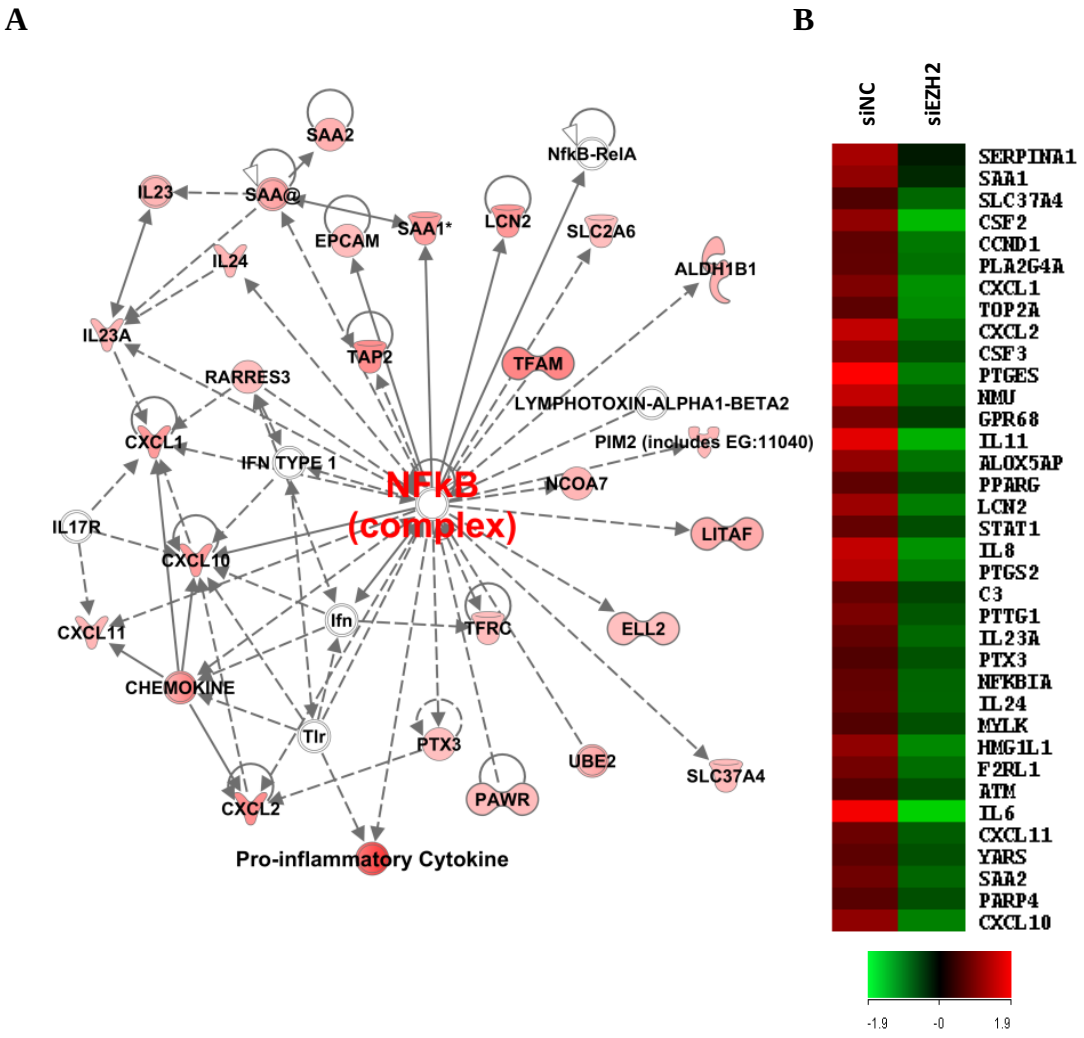

Figure 3.2 Inflammatory network and its related genes regulated by EZH2

- **A.** Inflammatory response network showing the connection to NF-κB. Red molecules in the network represented the genes that are downregulated upon EZH2 depletion in MB231 cells.
- **B.** Downregulation of the genes following EZH2 depletion shown in (A) was represented in the heatmap.

RelA and RelB are the two main transcription factors in the NF-κB pathway with transactivational activity and they serve to modulate the canonical and non-canonical NF-κB pathway respectively. Depletion of these transcription factors would allow the identification of their respective potential target genes in the context of our model cell line, MB231. Hence, in order to identify NF-κB target genes that are positively regulated by EZH2, we individually depleted RelA and RelB in MB231 cells and compared the gene expression profiles with that in EZH2-depleted cells. We identified 129 and 101 genes that were down-regulated following RelA and RelB knockdown (using 2-fold cut off), respectively. Surprisingly, we observed that there was a common set of 62 genes co-regulated by both RelA and RelB (Figure 3.3). This result was unexpected as it was previously reported that RelA and RelB govern the expression of distinct sets of targets that would lead to different responses upon activation. We also noticed that among RelA and RelB-regulated genes, 48 were concomitantly downregulated by EZH2 knockdown, showing that a significant set of NF-κB targets (approximately 28%) appeared to be down-regulated by EZH2 depletion ($p<0.001$). The overlap between the regulated genes further supports the notion EZH2 could positively modulating the expression of NF-κB (both RelA and RelB) targets in MB231 cells.

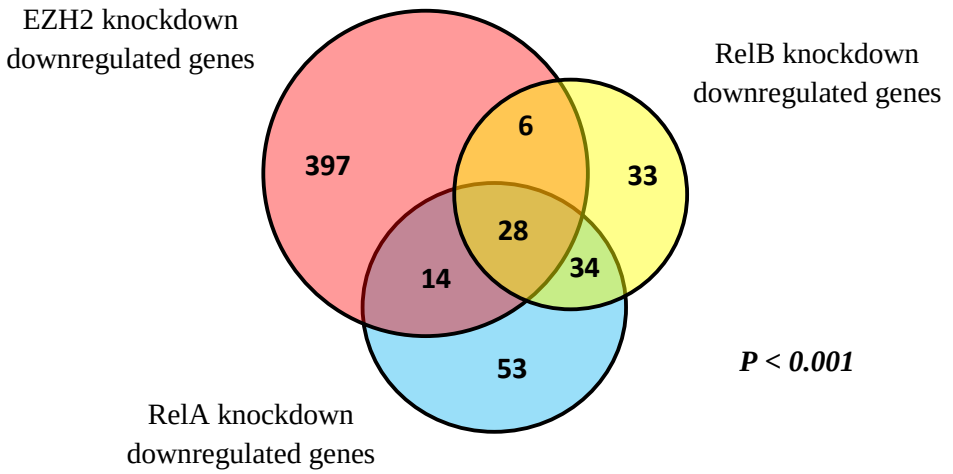

Figure 3.3 Overlap of EZH2-, RelA, and RelB-regulated genesets.

Venn diagram showing the overlapping of genes downregulated upon depletion of EZH2, RelA or RelB in MB231 cells.

The overlap of EZH2 and RelA/RelB regulated genes raised at least two possibilities: (i) EZH2 knockdown reduces RelA/RelB expression levels; (ii) EZH2 knockdown downregulates RelA/RelB transcriptional activity. To justify the effect of EZH2 knockdown on NF-κB expression or activity, we performed Western blot analysis and NF-κB-specific luciferase reporter assay in MB231 and another BLBC cell line, BT549. Based on the results, the first possibility was eliminated as EZH2 depletion affected neither RelA nor RelB protein expression levels (Figure 3.4A, Bottom). On the other hand, from the NF-κB reporter assay, EZH2 knockdown clearly reduced the reporter activity of NF-κB (Figure 3.4A, Top) in both MB231 and BT549 cell lines, showing that the effect was not specific and limited to a single cell line. As the positive controls, NF-κB reporter activities were reduced robustly upon RelA or RelB knockdown, indicating that the signals generated from the NF-κB reporter plasmid could reflect the transcriptional activity of both RelA and RelB. Noteworthy, the protein expression of RelB was significantly reduced after the depletion of RelA. This was not surprising as RelB is known to be a direct target of RelA (Bren *et al*., 2001). This could impose a problem in this project as any overlap between the effects of RelA and RelB depletion could be mediated through indirect effect exerted by RelA on RelB expression. Nevertheless, this problem could be partially addressed by performing RelA and RelB ChIP in the later part of this thesis.

In concordance with reduced NF-κB reporter activity following EZH2 depletion, expression levels of NF-κB targets *TNF* and *IL8* were markedly down-regulated in both MB231 and BT549 cells (Figure 3.4B). Increasing evidences have pointed to the importance of TNFα in promoting breast cancer aggressiveness (Acharyya et al., 2012; Li et al., 2012). Biologically, TNFα activates RelA activity by triggering the degradation of IκB and hence the translocation of RelA into the nucleus via the canonical pathway. As RelB is a target gene of RelA, TNFα treatment leads to induction of both RelA and RelB activity. Here, we showed that in addition to the basal activity of NF-κB, TNFα-induced NF-κB activation was also abolished by EZH2 knockdown in MB231 and BT549 cells (Figure 3.4C). Collectively, these

findings validated a positive role of EZH2 in modulating the NF-κB-dependent transcription in aggressive BLBC cells.

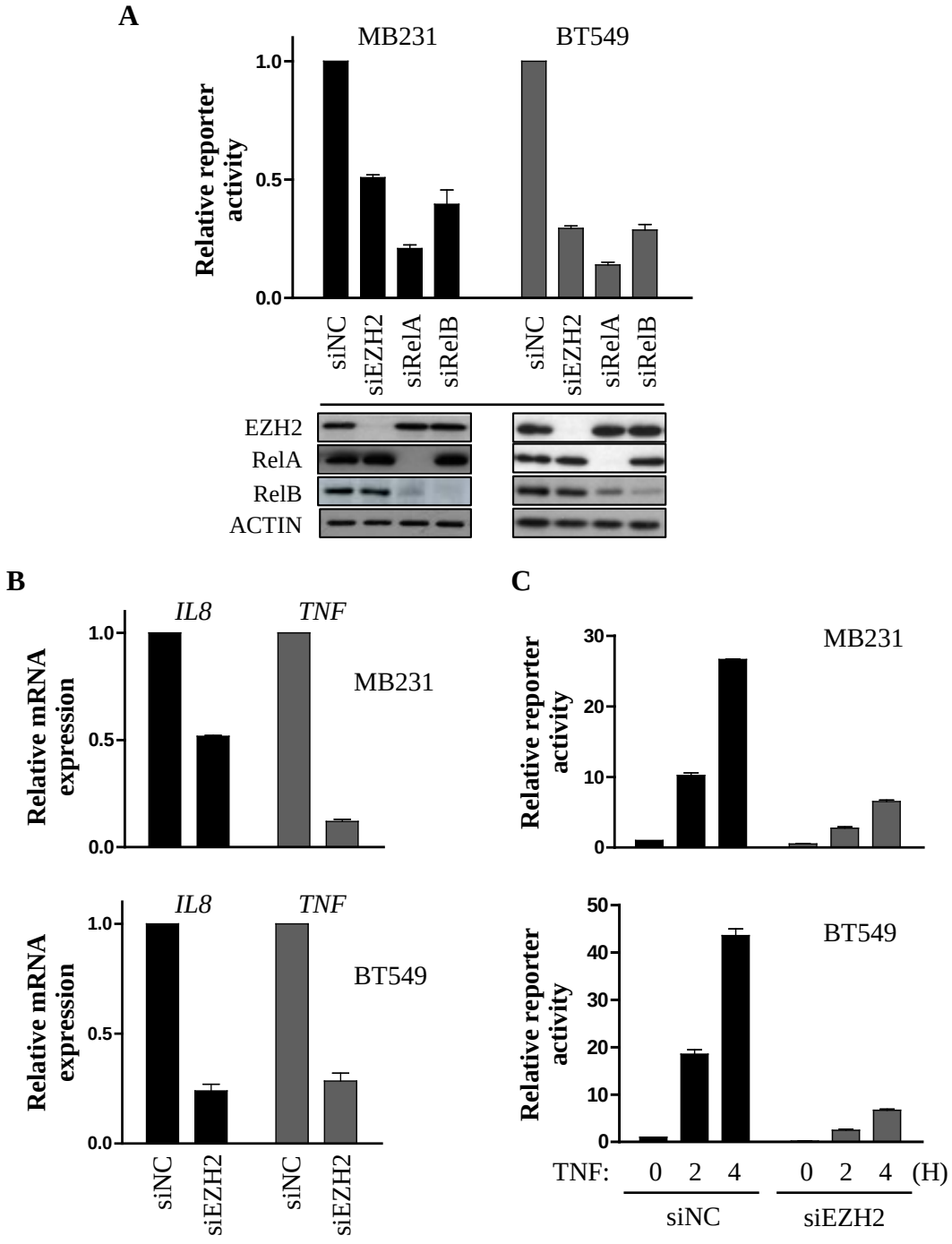

Figure 3.4 EZH2 depletion reduced NF-κB reporter activity.

A. NF-κB luciferase reporter activity in MB231 and BT549 cells upon knockdown of EZH2, RelA or RelB (Top). Knockdown efficiency was shown in immunoblot analysis (Bottom).
B. qRT-PCR assay results demonstrating relative mRNA expression levels of NF-κB target genes, *IL8* and *TNF*, in MB231 and BT549 cells upon EZH2 depletion.
C. NF-κB luciferase reporter activity in MB231 and BT549 cells upon EZH2 depletion and TNFα treatment for the indicated duration in hour (H).

3.2 EZH2 Positively Modulates NF-κB Target Gene Expression Independently of Histone Methyltransferase Activity.

The effect of EZH2 knockdown on NF-κB transcription activity could be due to the off-target effects of the siRNA used. To exclude this possibility, NF-κB reporter assay was repeated using another siRNA that targets the 5'UTR region of EZH2 mRNA. To further validate the specific effect of EZH2, we performed rescue assays restoring NF-κB reporter activity after EZH2 silencing by overexpressing wild-type EZH2 (EZH2 WT). Rescue assay is only possible when the siRNA used is unable to target the exogeneously expressed EZH2, which the mRNA transcript lacks of 5'- and 3'-UTR regions. As anticipated, the reduced NF-κB reporter activity resulting from the depletion of EZH2 using EZH2 5'UTR siRNA could be rescued by ectopic expression of wild type EZH2 in both MB231 and BT549 (Figure 3.5, Top). This result supports the specificity of EZH2 in regulating NF-κB activity. The incomplete rescue of NF-κB activity by EZH2 WT could be due to the low efficiency of transient overexpression as such transfection could normally take effect on only about 30% of cell population. Although the protein level of the ectopic EZH2 WT appeared higher than the untransfected control (Figure 3.5, Bottom), that could be a result of amplification of exogeneous gene product from the 30% of transfected cells.

EZH2 SET domain is required to induce gene silencing by catalyzing H3K27 trimethylation. We next asked whether such a catalytic function is required for EZH2 to positively regulate NF-κB-dependent transcription. We compared the abilities of EZH2 WT and EZH2 SET domain deletion mutant (EZH2 SETΔ) in rescuing the downregulation of NF-κB activity in EZH2-depleted cells. In both MB231 and BT549 cells, depletion of the endogenous EZH2 and the associated H3K27me3 by this siRNA were effectively rescued by the ectopic expression of the EZH2 WT but not EZH2 SETΔ, demonstrating the functionality of these overexpression constructs (Figure 3.5, Bottom). Interestingly, overexpression of EZH2 SETΔ, which lacked the methyltransferase activity for H3K27me3, was still able to

strongly restored NF-κB activity after EZH2 knockdown (Figure 3.5, Top), suggesting a SET domain-independent function of EZH2 on modulating NF-κB activity. Notably, ectopic EZH2 SETΔ consistently displayed higher levels of protein accumulation compared to the ectopic EZH2 WT, this is in accordance with the stronger rescue of NF-κB activity by EZH2 SETΔ compared to EZH2 WT (Figure 3.5, Bottom).

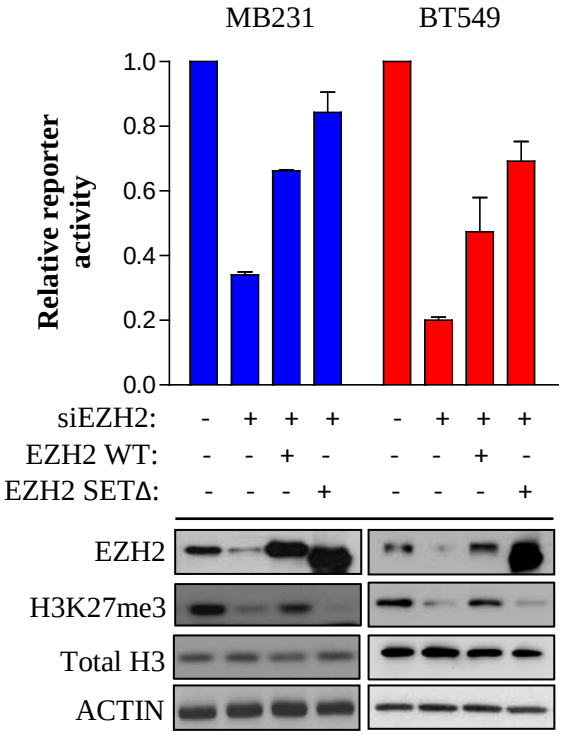

Figure 3.5 EZH2 WT and SETΔ rescued NF-κB reporter activity.

NF-κB luciferase reporter activity in MB231 and BT549 transfected with EZH2 5'-UTR siRNA followed by over-expression of EZH2 WT or SETΔ (Top). Western blot analysis of the indicated samples (Bottom).

To further substantiate the evidences of SET domain-independent function of EZH2 in regulating NF-κB activity, EZH2 WT and EZH2 SETΔ were stably expressed in an immortalized human mammary epithelial cell line, HMEC, generated through retroviral infection. In consistent with the results of transient overexpression, stable overexpression of EZH2 SETΔ resulted in a robust induction of NF-κB reporter activity (Figure 3.6A, Top). However, stable overexpression of EZH2 WT had only a modest effect, probably due to insufficient overexpression of EZH2 (Figure 3.6A). Concomitantly, the expression of of NF-κB targets like *TNF*, *IL6*, and *IL8* was induced strongly in EZH2 SETΔ stable cell line (Figure 3.6B).

Notably, ectopic expression of EZH2 SETΔ depletes the protein expression of endogeneous EZH2 and the corresponding H3K27me3 mark through an unknown mechanism (Figure 3.6A, Bottom). The stronger enhancing effect of EZH2 SETΔ on NF-κB transcription activity could imply a scenario whereby the SET-domain independent function is enriched in EZH2 SETΔ-expressing cell line when compared to the EZH2 WT-expressing cell line as the EZH2 WT variant could be potentially recruited and engaged in PRC2-mediated activity rather than the SET-domain independent function. Collectively, these findings support the hypothesis in which EZH2-mediated activation of NF-κB signaling does not require histone methylation catalytic activity.

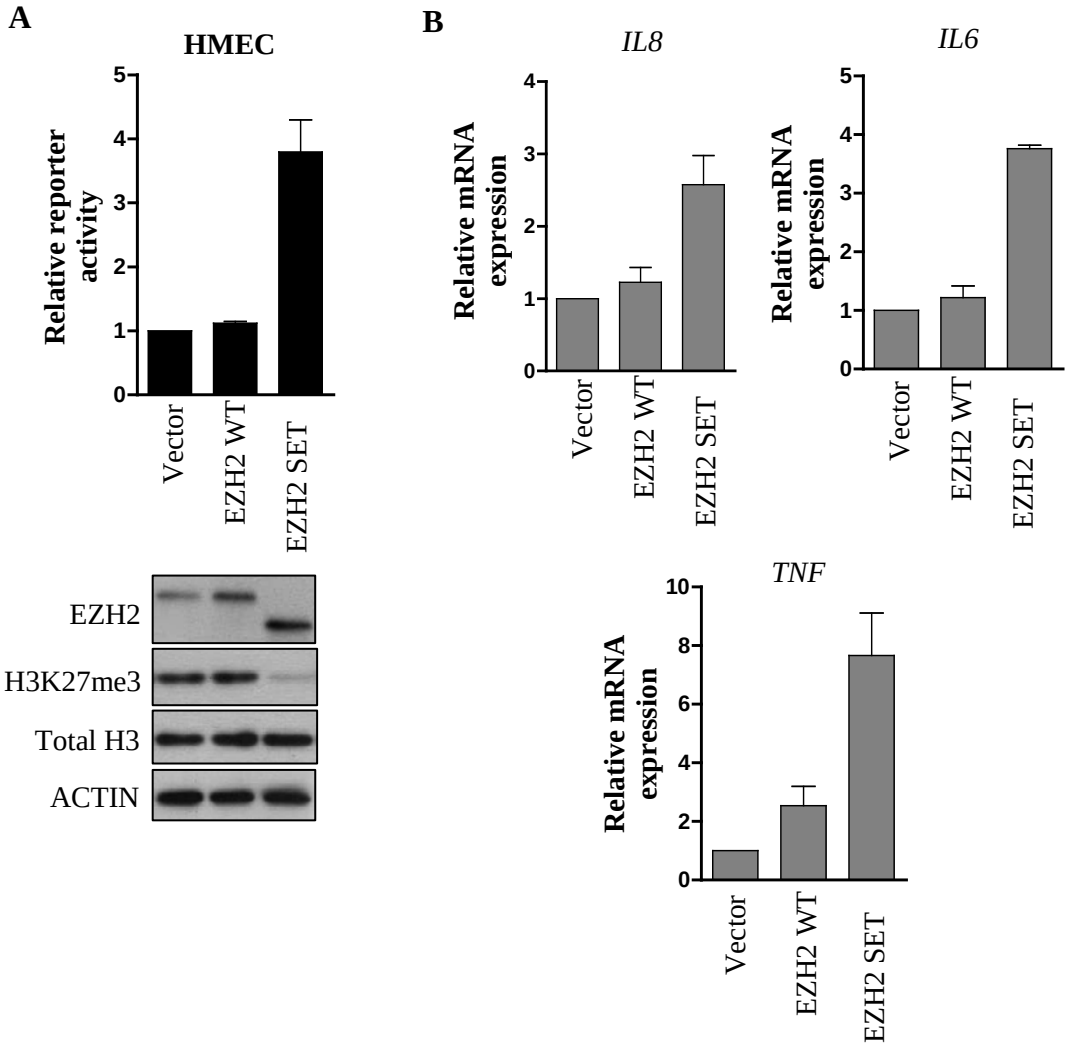

Figure 3.6 Stable overexpression of EZH2 WT and SETΔ induced NF-κB activity.

A. NF-κB luciferase reporter activity in HMEC cells overexpressing EZH2 WT or SETΔ (Top). Western blot analysis of indicated samples (Bottom).
B. RT-PCR analysis of *IL6*, *TNF*, and *IL8* in HMEC cells overexpressing EZH2 WT or SETΔ.

3.3 EZH2 Forms a Ternary Complex with RelA and RelB in Aggressive Breast Cancer Cells

Since EZH2 could modulate NF-κB activity independent of the SET domain function, these findings brought up a possibility that EZH2 may directly regulate NF-κB signaling. To explore this possibility, we began to ask whether EZH2 regulates NF-κB signaling through physical association with RelA or RelB, the two main transactivational members of NF-κB. To examine this hypothesis, we performed co-immunoprecipitation (Co-IP) in MB231 with or without the stimulation of TNFα. In each respective immunoprecipitates of RelA, RelB, or EZH2, we were able to detect the other two proteins, which were further enriched upon TNFα-treatment (Figure 3.7A). Notably, RelA was found to be co-immunoprecipitated with RelB, consistent with a previous report (Jacque *et al*., 2005). These results indicate that endogenous EZH2 forms a complex with RelB and RelA in MB231 cells. Yet, while SUZ12, a component of PRC2 (mainly contains EZH2, SUZ12 and EED), was found in EZH2 immunoprecipitates, it was not detected in RelB or RelA immunoprecipitates. This proposed the ability of EZH2 to form two separate protein complexes in MB231 cells: (i) PRC2; (ii) EZH2/RelB/RelA. Densitometry analysis of Western blot showed that approximately 1-2% of total EZH2 was associated with RelA/RelB and 4-5% of RelA/RelB interacted with EZH2 (Figure 3.7B).

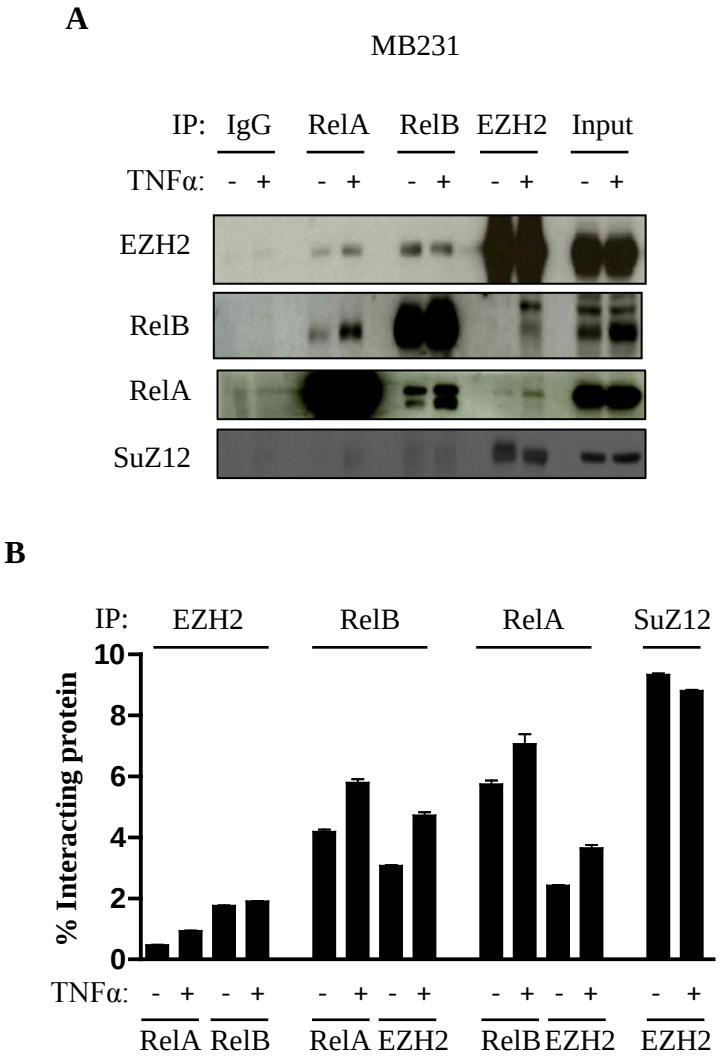

Figure 3.7 EZH2 physically interacted with RelA and RelB endogeneously.

Co-IP assay using nuclear extract of MB231 cells stimulated with TNFα for 2 hours (Top). The bands were technically quantified three times by densitometry (Bottom).

Given that EZH2 SETΔ could rescue the transcriptional activity of NF-κB, we hypothesized that the association of EZH2 with RelA/RelB is independent of the SET domain. To test this hypothesis, we overexpressed myc-tagged EZH2 WT or EZH2 SETΔ in the presence of RelA and RelB overexpression in HEK293T cells. HEK293T cells are immortalized human embryonic kidney cells, which stably expresses SV40 Large T-antigen that enables episomal replication and subsequent amplification of transfected plasmids consisting of SV40 replication origin. As a result of plasmid amplification, genes that are cloned into the plasmids would be transcribed at a high efficiency. Hence, HEK293T was chosen for transient overexpression of genes of interest. In this experiment, we adopted HEK293T for transient simultaneous overexpression of EZH2, RelA and RelB in order to study the potential physical interactions between these players. We perform individual immunoprecipitation of EZH2, RelA, and RelB using their respective specific antibodies and we observed that RelB was detected in both EZH2 WT and EZH2 SETΔ immunoprecipitates, which the interactions were more evident upon TNFα stimulation (Figure 3.8). Compared to RelB, RelA appeared to be less interactive with EZH2 and their interaction was only evident in EZH2 SETΔ immunoprecipitates, suggesting that EZH2 may preferentially associate with RelB in this experimental condition. Notably, RelA also interacted with RelB in a TNFα stimulation dependent manner. Interestingly, the interaction seemed to be stronger in the presence of EZH2 WT or EZH2 SETΔ. Such differences were more evident in RelA precipitates. This infers that the presence of EZH2 WT or EZH2 SETΔ may enhance the interaction between RelA and RelB.

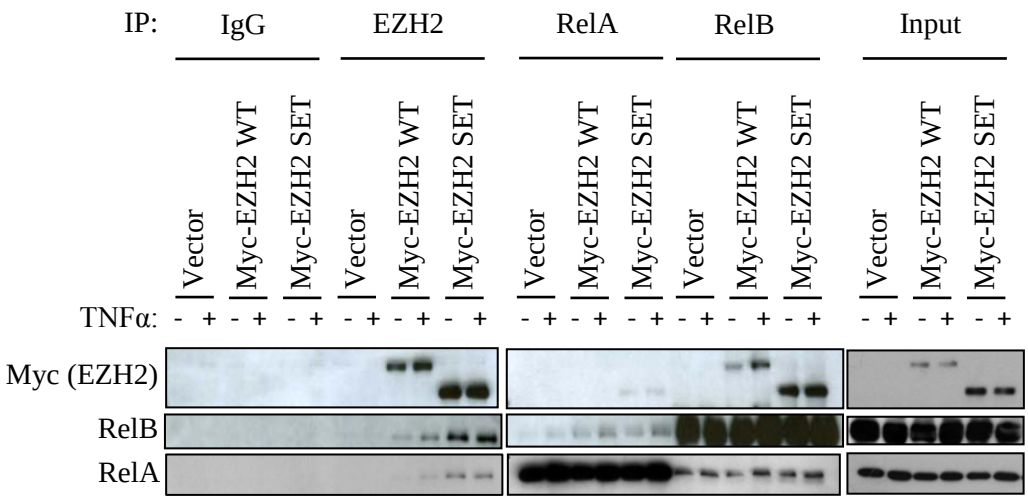

Figure 3.8 EZH2 WT and SETΔ physically interacted with RelA and RelB.

Co-IP assay using nuclear extract of 293T cells over-expressed with RelA, RelB, and myc-tagged EZH2 constucts followed by TNFα stimulation for 2 hours.

To demonstrate a direct interaction between EZH2 and RelA/RelB, we performed an *in vitro* pull down assay using bacterial expressed recombinant proteins. As shown in Figure 3.9A, both MBP-tagged RelA or RelB recombinant fusion proteins could associate with GST-tagged EZH2. MBP protein served as a negative control in the pull down. This indicated that EZH2 could directly interact with RelA and RelB and such interaction did not require the presence of other components in mammalian systems. Nevertheless, this pull down experiment could suggest two different scenarios: (i) EZH2 interacts with both RelA and RelB in the same complex, and; (ii) EZH2 interacts with RelA and RelB in two separate complexes. To examine the first scenario, we designed a re-IP assay. We first performed *in vitro* pull down of the GST-tagged EZH2 followed by the elution of the pull down complexes using reduced glutathione. The eluates were then subjected to a sequential co-immunoprecipitation of the second component (i.e. RelA) and attempt to detect the third component (i.e. RelB) in the complex. If EZH2 interacts with RelA and RelB to form independent complexes, the third component would not be enriched during the second immunoprecipitation assay. To facilitate the formation of ternary complex, the MBP tags of RelA and RelB were removed by TEV protease treatment in order to reduce steric hindrance. Consistent with the observation in Figure 3.9A, both RelA and RelB were enriched in the pull down of GST-EZH2 but not the negative control, GST (Figure 3.9B). When we further subjected the eluates of GST pull down to RelA-specific immunoprecipitation, RelB was only detected in GST-EZH2 pull down sample, but not in either the IgG counterpart or GST control pull down (Figure 3.9B). This suggests that the EZH2-interacting RelA could simultaneously associates with RelB in the same complex.

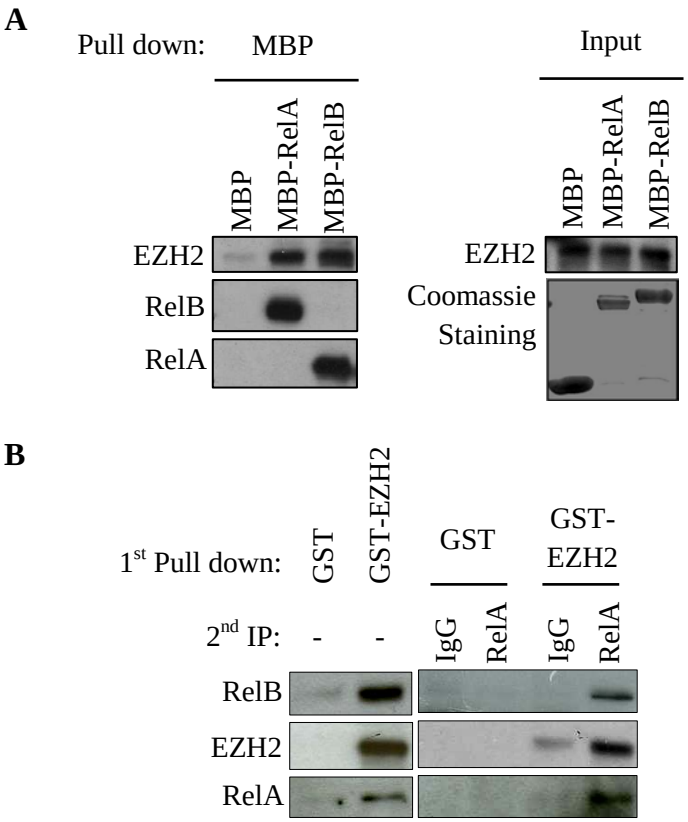

Figure 3.9 EZH2, RelA, and RelB direct interacted with each other to form a ternary complex.

A. *In vitro* pull down assay using recombinant MBP-RelA and MBP-RelB as well as GST and GST-EZH2 proteins.
B. *In vitro* re-immunoprecipitation assay using recombinant RelA and RelB as well as GST and GST-EZH2 proteins. GST or GST-EZH2 and its interacting partners were affinity purified as described in experimental procedures and further immunoprecipitated using RelA-specific or control IgG and subjected to Western blot analysis.

To demonstrate the presence of the ternary complex endogeneously in cells, re-IP is not plausible as competitive peptides are not available for elution of the complex from the first pull down process. Alternative elution approaches such as detergent-containing buffer and high temperature were also not feasible as these conditions would probably disrupt the ternary complex. Thus, to investigate the presence of endogeneous ternary complexes, we performed a knockdown IP experiment to assess the effect of the depletion of one component on the interaction between the other two components. As a result, the depletion of endogenous RelB greatly reduced the interaction between endogenous EZH2 and RelA in MB231 cells, suggesting that the presence of RelB is important for EZH2-RelA complex formation (Figure 3.10). One possible explanation could be RelB is stabilizing EZH2-RelA interaction by direct association with the complex. Taken all together, these biochemical data indicate the formation of a ternary complex between endogenous EZH2, RelB and RelA in MB231 cells and provide a potential mechanistic link to the positive regulation of NF-κB activity by EZH2 in BLBC.

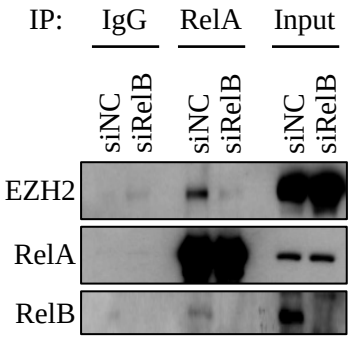

Figure 3.10 RelB depletion disrupted EZH2-RelA interaction.

Co-IP was performed on MB231 depleted of RelB and treated with TNFα for 2 hours.

3.4 EZH2 and RelA/RelB Co-Regulate a Subset of NF-κB Targets by Inter-Dependent Promoter Occupancy

Given that EZH2 may regulate NF-κB target genes through forming a complex with RelA and RelB, which both have known oncogenic roles in aggressive breast cancers (Helbig *et al*., 2003; Wang *et al*., 2007), we next sought to identify EZH2/RelA/RelB-co-regulated NF-κB targets that might be associated with aggressive phenotype of BLBC. Using non-supervised hierarchical clustering of genes identified through gene expression profiling of MB231 cells that were depleted of EZH2, RelA or RelB (2-fold cut-off) with or without TNFα stimulation, two distinct clusters of genes were revealed: (i) one cluster of genes (n=46) appeared to be regulated by all three components of EZH2, RelA, and RelB and showed a strict dependency on EZH2, RelA, and RelB upon TNFα treatment while (ii) another cluster of genes (n=6) seemed to be regulated mainly by RelA and was capable to be induced by TNFα when RelB or EZH2 was depleted, but remained downregulated when RelA was depleted (Figure 3.11A). Notably, most of the genes from the second cluster geneset were downregulated at the basal level upon EZH2 and RelB depletion, possibly due to the reduction of many cytokines such as TNFα and IL6 that could otherwise drive autocrine responses of NF-κB signaling. Short-term TNFα treatment was shown to activate RelA more rapidly and more robustly without much influence on RelB activity (Derudder *et al*., 2003). This allows the distinction of RelA and RelB target genes. Hence, we speculated that when TNFα was added exogeneously, endogeneous RelA could be stimulated and the expression of the target genes that were mainly regulated by RelA could be restored even if EZH2 or RelB was depleted. This trend of regulation was further validated by RT-PCR analysis of representative genes from each cluster (Figure 3.11B).

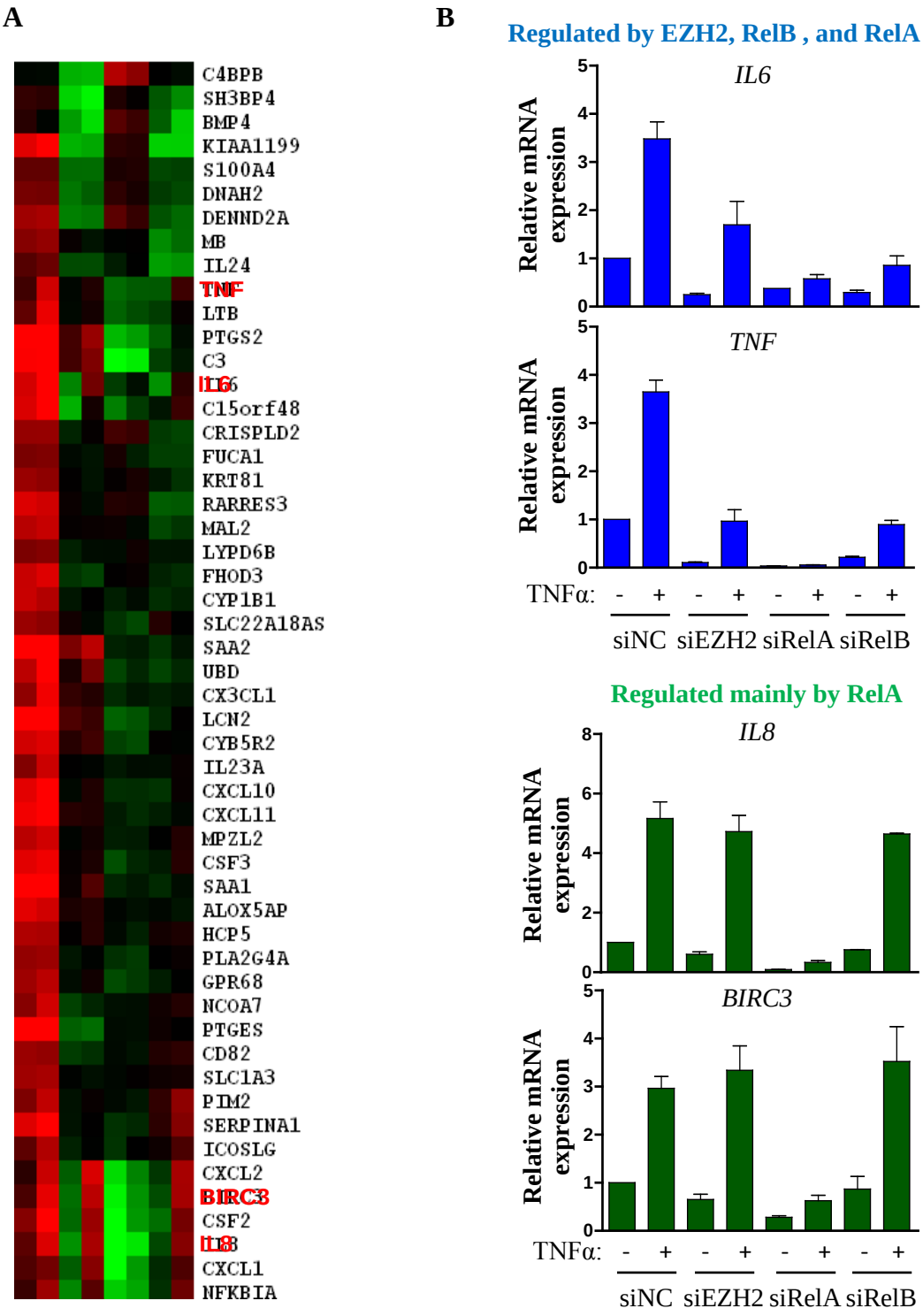

Figure 3.11 Two clusters of EZH2/NF-κB regulated genes.

A. Non-supervised hierarchical clustering of NF-κB target genes that were co-regulated with EZH2. Gene sets were obtained from microarray gene expression profiling of MB231 cells depleted of EZH2, RelA, and RelB followed by TNFα treatment for 4 hours.
B. RT-PCR analysis of representative genes from each cluster of (A).

The dependence of *IL6* or *TNF* expression on RelB was further verified by stable overexpression of RelB in MB231 cells. RT-PCR analysis showed that *IL6* or *TNF* expression was robustly elevated upon ectopic expression of RelB, whereas the expression levels of these genes was less responsive to ectopic RelA overexpression (Figure 3.12). Noticeably, EZH2 remained essential in regulating *IL6* and *TNF* expression despite of the overexpression of RelB or RelA. In contrast, *IL8* and *BIRC3* from the second cluster geneset were neither responsive to ectopic RelB, nor altered after EZH2 knockdown upon TNFα treatment (Figure 3.12). In concordance with the hypothesis that the second cluster geneset was mainly regulated by RelA, exogeneous expression of RelA was sufficient to induce the expression of *IL8* and *BIRC3* to at least by two-fold. This elevation of expression was less obvious under TNFα stimulation possibly because RelA overexpression was adequate to turn on the expression of its target genes. Taken together, these findings support that EZH2-regulated NF-κB targets such as *IL6* and *TNF* from the first cluster are EZH2-, RelA- and RelB-dependent, while the second cluster of NF-κB target genes such as *IL8* and *BIRC3* are not dependent on RelB and EZH2. The model of the two types of regulation was illustrated in Figure 3.13.

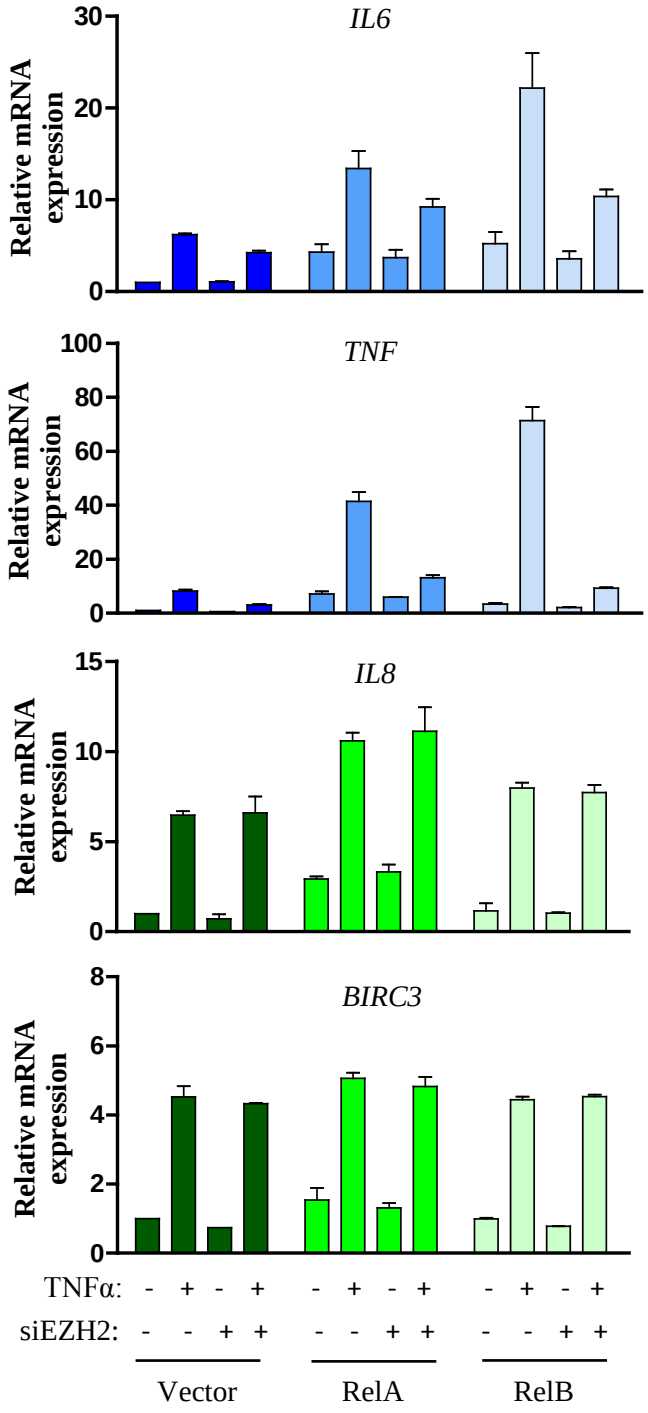

Figure 3.12 Ectopic expression of RelA and RelB induced the expression of different subsets of genes.

RT-PCR analysis of representative genes in MB231 cells overexpressing RelA or RelB followed by depletion of EZH2 with or without TNFα treatment for 4 hours.

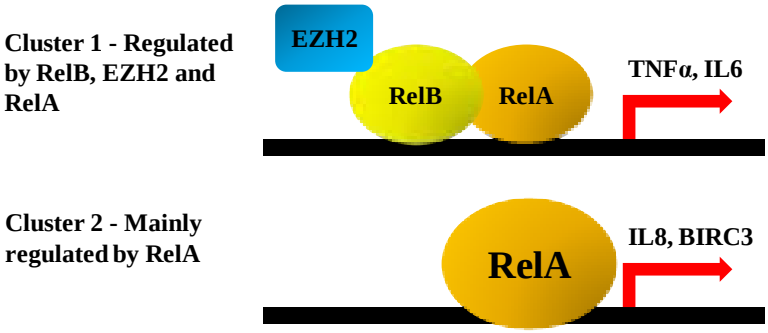

Figure 3.13 A Model proposed two modes of transcription regulation by EZH2 and NF-κB.

A model proposed the two modes of transcription regulation of the target genes by EZH2 and/or NF-κB.

To understand the mechanisms by which EZH2 selectively cooperates with RelA and RelB towards NF-κB target gene regulation, we next analyzed the promoter binding of EZH2, RelB, and RelA by performing chromatin immunoprecipitation (ChIP) in TNFα-treated MB231. Binding signals were then detected using a panel of PCR primer pairs that spanned the gene promoters of *IL6*, *TNF*, *IL8* and *BIRC3*. Significantly, all three proteins were found to be highly enriched in both *IL6* and *TNF* promoters with binding peaks around the 1000-500bp upstream of the transcription start sites (Figure 3.14). However, despite a high EZH2 enrichment, no corresponding H3K27me3 enrichment was detected on *IL6* and *TNF* promoter, further supporting a histone methylation-independent activity of EZH2. In contrast, *IL8* and *BIRC3* promoters only displayed high RelA binding, but not EZH2 and RelB binding. These observations corresponded well with the hypothetical model illustrated in Figure 3.13.

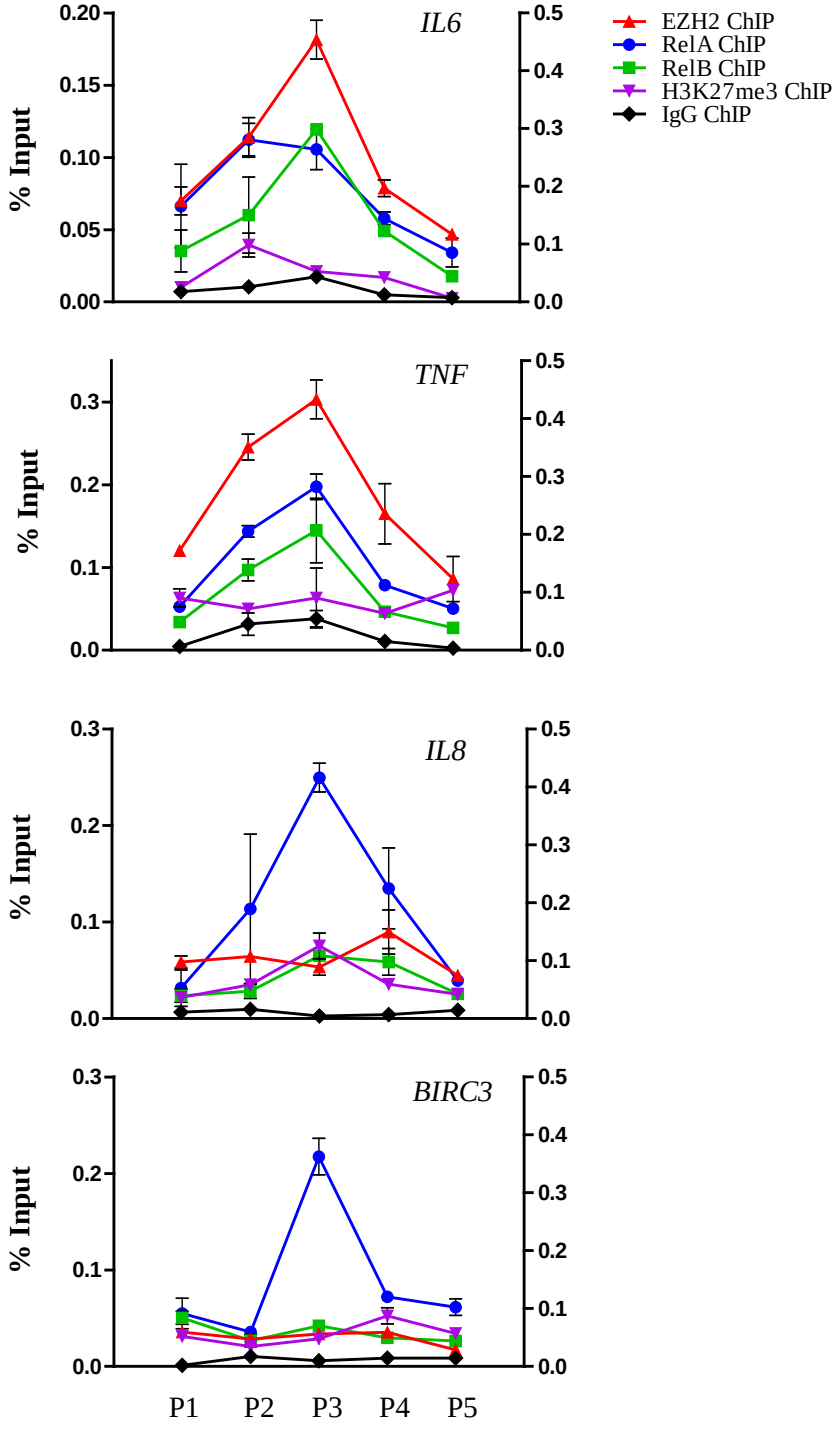

Figure 3.14 Promoter occupancy of EZH2, RelA, and RelB in MB231.

ChIP assay of EZH2, RelA, RelB, and H3K27me3 on the promoters of *TNF*, *IL6*, *IL8*, and *BIRC3* in MB231 cells treated with TNFα for 2 hours. Primer pairs encompass a 2.5 kb surrounding the TSS (transcription start site) for *TNF*, *IL8*, and *BIRC3* with approximately 500 bp interval. P3 is located in the region within 500bp upstream of TSS. P4 is located the region within 500bp upstream of TSS for *IL6*. The percent input of H3K27me3 ChIP was plotted on the right y-axis. Quantification of binding was determined as a percent of input DNAs.

Recall in Figure 3.10, depletion of RelB affected the complex formed between RelA and EZH2. If first cluster geneset was dependent of the ternary complex of EZH2/RelA/RelB, disruption of the complex by depleting any of the three components could affect the DNA binding of the other two. To investigate this likelihood, knockdown ChIP assay was performed. Indeed, TNFα-induced recruitments of RelA and RelB to *IL6* and *TNF* promoters were markedly reduced by EZH2 knockdown (Figure 3.15). Similarly, knockdown of RelB decreased the recruitment of EZH2 and RelA. Importantly, the recruitment of RelA on the promoters of *IL8* and *BIRC3*, which were void of EZH2 and RelB binding, was not influenced by EZH2 or RelB knockdown, indicating that the reduction of RelA recruitment to promoters of *IL6* and *TNF* after EZH2 or RelB knockdown was not a general effect to all RelA target genes. These results indicate that the recruitment of EZH2, RelA, and RelB to *IL6* and *TNF* gene promoters are inter-dependent. In contrast, EZH2 or RelB knockdown had no effect on RelA binding to *IL8* and *BIRC3* promoters. These results further support that the genes from cluster two (Figure 3.11B) were mainly regulated by RelA and were not directly regulated by EZH2 and RelB.

Albeit knockdown ChIP has demonstrated the interdependency of EZH2, RelA, and RelB occupancy at *IL6* and *TNF* promoter, there is still insufficient evidence to show co-occupancy of all three factors at the promoter. Thus, extensive pair-wise sequential ChIP was carried out. Following the EZH2 first ChIP, both RelA and RelB binding was enriched in EZH2-bound *IL6* and *TNF* promoters, but not in *IL8* and *BIRC3* promoters (Figure 3.16), indicating that RelA/RelB were concurrently bound to these promoter with EZH2. Comparable results were also obtained in RelA or RelB first ChIP experiments. Nevertheless, pairwise sequential ChIP could only showed the co-occupancy of two factors. Binding of the ternary complex could hardly be demonstrated as triple sequential ChIP was technically unfeasible. Taken together, we conclude that EZH2, through interacting with RelA and RelB, is required for the activation of a subset of NF-κB target genes in BLBC cells. This established a potential new function of EZH2 acting as a transcriptional activator in BLBC.

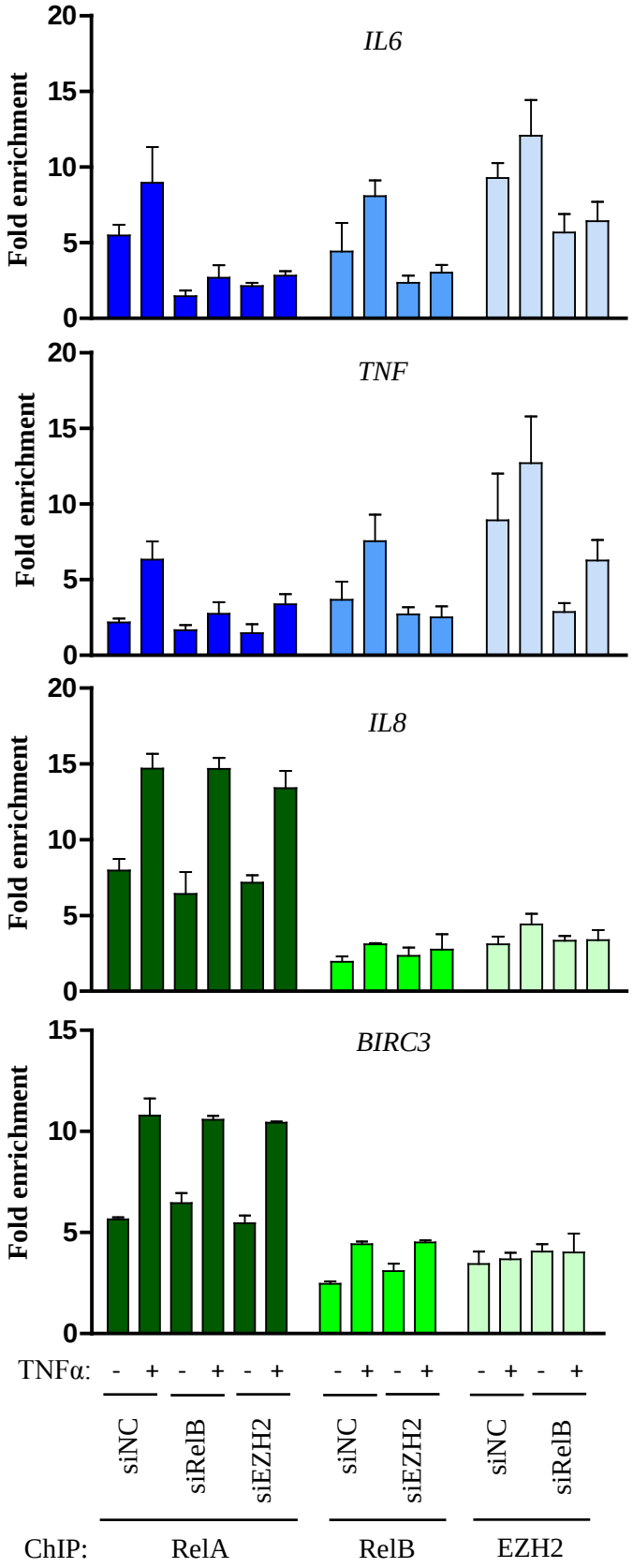

Figure 3.15 Knockdown-ChIP of EZH2, RelA, and RelB in MB231.

ChIP assay of EZH2, RelA or RelB on the promoter of *TNF*, *IL6*, *IL8*, and *BIRC3* genes in MB231 cells depleted with EZH2 or RelB, followed by TNFα treatment for 2 hours. Quantification of binding was represented as fold enrichment over IgG.

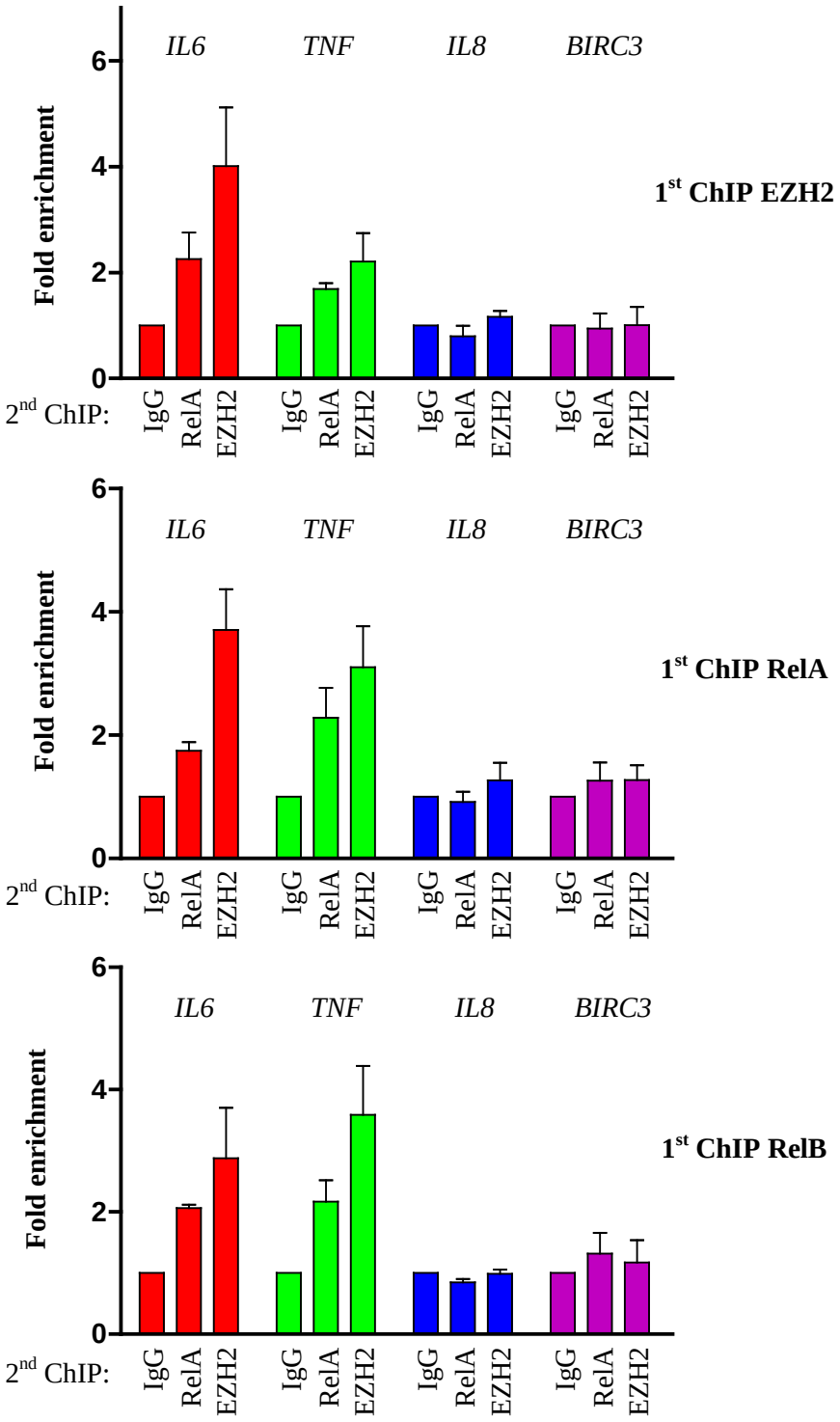

Figure 3.16 Sequential ChIP of EZH2, RelA, and RelB in MB231.

Sequential ChIP assay was performed to assess *in vivo* co-occupancy of EZH2, RelA, and RelB on the chromatin. First ChIP and second ChIP antibodies were indicated as the chart title and x-axis labels. Quantification of binding was represented as fold change to control IgG second ChIP.

3.5 NF-κB Target Gene Signature Co-Regulated by EZH2, RelA, and RelB Discriminates Basal vs Luminal Subtype of Breast Cancers and is Associated with Poor Disease Outcome

RelB was reported to be aberrantly expressed in BLBC. In this study, we demonstrated the importance of RelB for EZH2-RelA interaction in the BLBC cell line, MB231. Hence, the overexpression of RelB could be the cause that allows the formation of EZH2/RelA/RelB complex, leading to the subsequent increased expression of target genes regulated by this complex. To determine whether EZH2/RelA/RelB co-regulated NF-κB target geneset (n=46) identified in MB231 cells are also associated with other BLBC cell lines, we interrogated the published gene expression dataset generated from 54 breast cancer cell lines, including 26 basal and 25 luminal (and 3 uncharacterized) (Neve *et al*., 2006) and performed non-supervised clustering analysis based on this geneset. The expression of ER and its essential cofactors, FOXA1 and GATA3 were shown to validate the luminal or BLBC status of the cell lines. Strikingly, EZH2 and NF-κB-coregulated gene list was able to efficiently separate basal from luminal type breast cancer cells with approximately 90% proper segregation (Figure 3.17).

Although the 46 genes were demonstrated to be coregulated by EZH2/RelA/RelB in MB231, it was understandable that not all of these genes would remain coregulated in other BLBC cell lines due to different cellular context. Based on the clustering of EZH2/RelA/RelB coregulated genes, we identified a cluster of 12 genes that were consistently expressed higher in BLBC compared to the luminal breast cancer cell lines at both individual gene and average gene expression levels (p=0.0001) (Figure 3.17 and 3.18A). This indicates that deregulation of these genes is not just limited to MB231 cells, but common to BLBC cell lines in general. Notably, gene set comprising of these 12 genes include *IL6*, *SAA1*, and *PTGS2* (encoding Cox2) that have been previously shown to be associated with aggressive breast cancers and poor disease outcome (Pierce *et al*., 2009; Sansone *et al*., 2007; Zhao *et al*., 2008).

To further establish the clinical significance of this finding, we carried out an expression analysis of these 12 genes using two additional microarray datasets from Farmer and Netherlands breast cancer cohorts (Bos *et al*., 2009; Farmer *et al*., 2005) that were downloaded from Oncomine. Consistent with the analysis in the cell line dataset, the averaged expression of the 12 signature genes were significantly higher in basal or ER negative breast cancer samples as compared to luminal or ER positive breast tumors in these two cohorts of tissue samples (Figure 3.18B and 3.18C). The expression of the 12 signature genes was inversely correlated with the expression of *ER*, *FOXA1* and *GATA3*, markers of luminal breast cancer.

Interestingly, the expression of RelB but not RelA was consistently higher in BLBC or ER-negative breast cancer cells in all the three datasets analyzed (Figure 3.19), in concordance with the higher expression of the 12 signature genes. Furthermore, based on the three datasets, only EZH2 showed consistent overexpression in ER-negative breast cancer or BLBC but not the other two core components of PRC2. These support our model that instead of PRC2 complex, SET domain independent function of EZH2 might be primarily responsible for the transactivation of NF-κB activity.

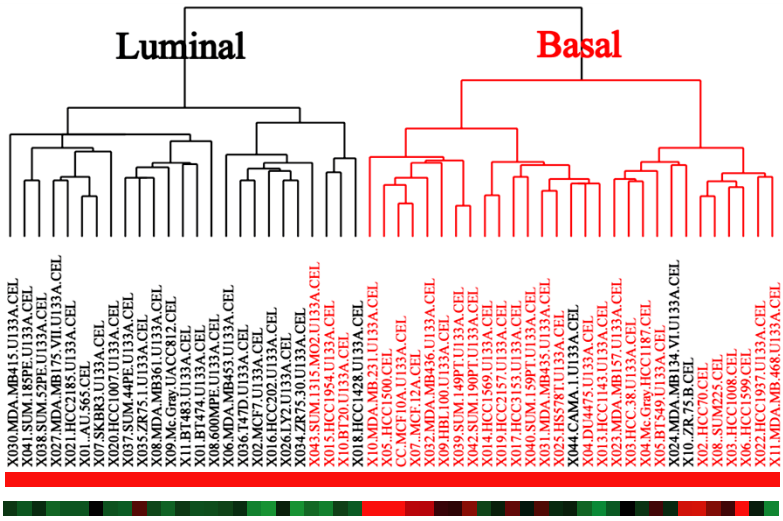

Figure 3.17 Hierarchical clustering of breast cancer cell lines based on expression of EZH2/NF-κB coregulated genes.

Non-supervised clustering of 54 breast cancer cell lines based on 46 genes that were co-regulated by EZH2, RelB, and RelA. A subset of 12 genes showing higher expression in basal- vs luminal- breast cancer cell lines in the heatmap. Expressions of ER associated genes (*ESR1*, *FOXA1* and *GATA3*), PRC2 components (*EZH2*, *EED*, and *SUZ12*) as well as *RELA* and *RELB* were also shown

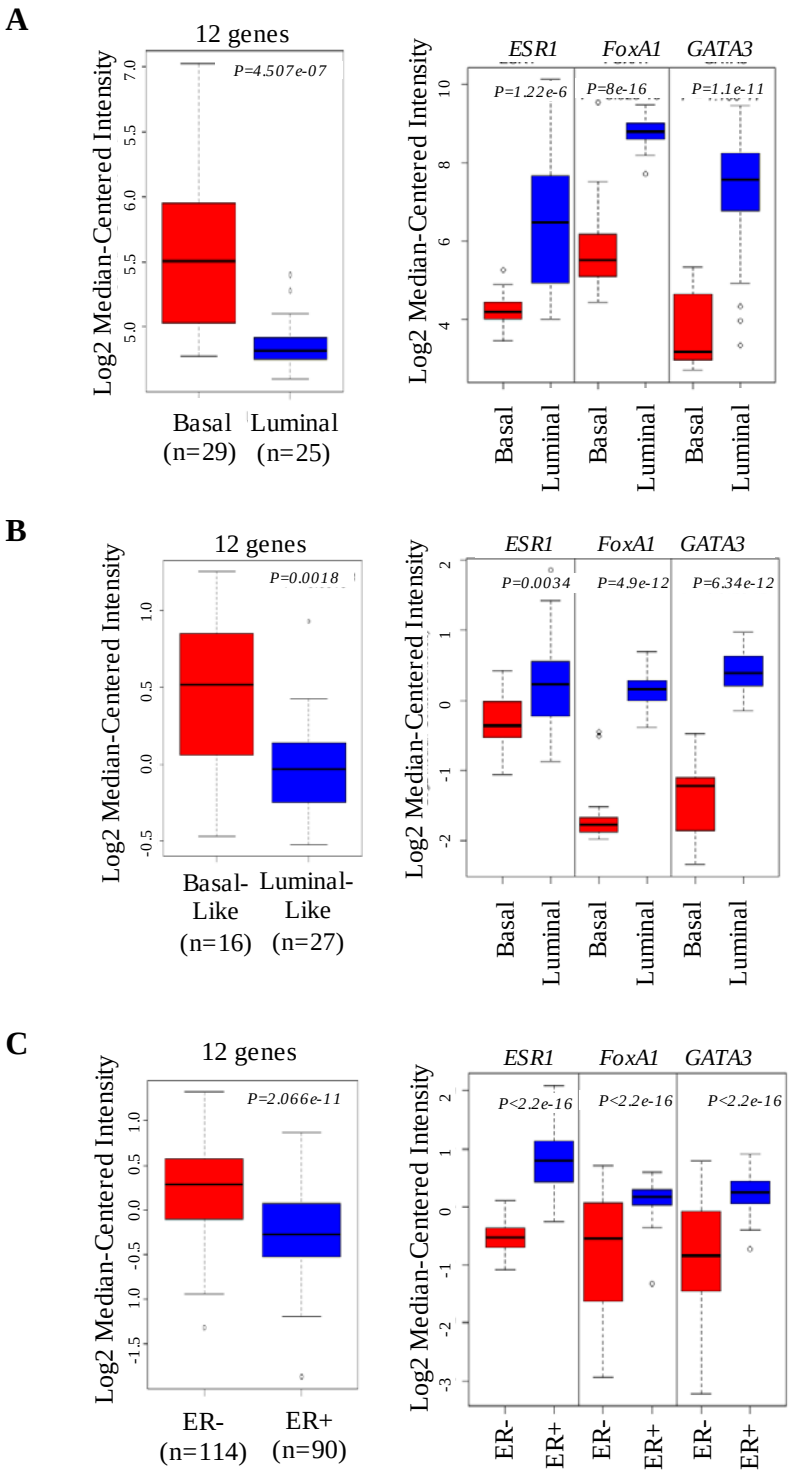

Figure 3.18 Expression of 12 signature genes and ER associated genes.

Box plots showing the average expression level of the 12 signature genes and ER associated genes in basal and luminal breast cancer cell lines (A); patient tissue in Farmer cohort (B); and patient tissue in Netherlands cohort (C).

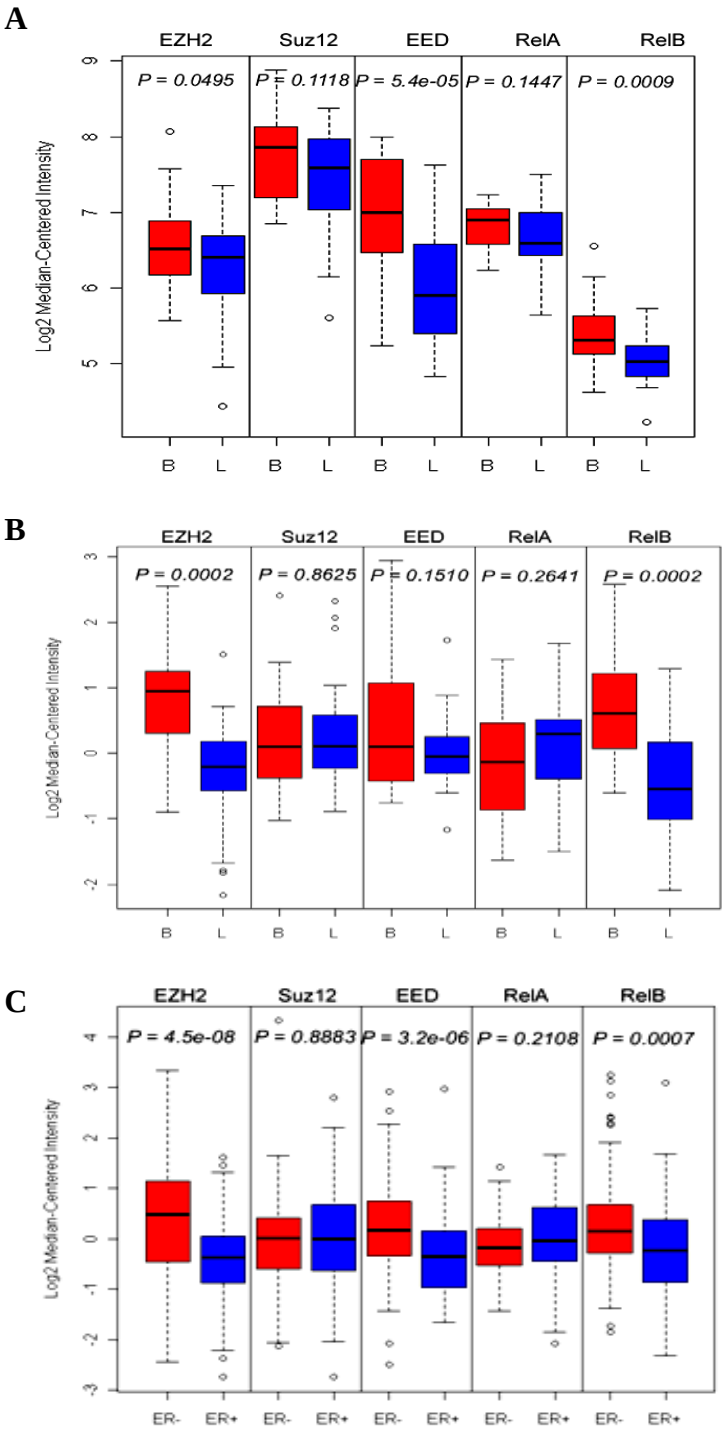

Figure 3.19 Expression of RelA, RelB, EZH2 and other two PRC2 components.

Box plots showing the expression level of PRC core components (*EZH2*, *SUZ12*, and *EED*) as well as *RelA* and *RelB* in basal and luminal breast cancer cell lines (A); patient tissue in Farmer cohort (B); and patient tissue in Netherlands cohort (C). Note: B=BLBC; L=Luminal breast cancer.

More importantly, Kaplan-Meier survival analysis also demonstrated a prognostic value of the 12 signature genes in breast cancer patients of Netherlands cohort. Specifically, when we classified the patients based on the mean value of the average expression levels of the 12 genes, the breast cancer patients with higher expression of this geneset showed lower brain- and lung- metastasis-free survival probabilities in Kaplan-Meier plots with P=0.0188 and P=0.0152, respectively (Figure 3.20A). When we further stratified the patients based on quartile expression of the 12 genes, more significant differences in brain- and lung-metastasis-free survival probabilities were observed with P=0.0025 and P=0.0065, respectively (Figure 3.20B). This indicates that the clinical outcome correlates well with the expression levels of the 12 signature genes. Intriguingly, no difference was observed in bone metastasis-free in these patients regardless of the method of stratification either based on mean or quartile expression. This result was coincidental with the clinical observation that the preferred sites of distal metastasis in BLBC patients are brain and lung, but not bone. As a control, NF-κB targets that are not regulated by EZH2 or RelB were not found to be associated with brain or lung metastasis-free survival (Figure 3.21). Taken together, these results indicate that the expression levels of the 12 genes inversely correlated with the brain- and lung- metastasis-free survival probabilities of breast cancer patients. This highlights the clinical relevance of EZH2/RelA/RelB-dependent gene transcription in promoting the aggressiveness of breast cancer.

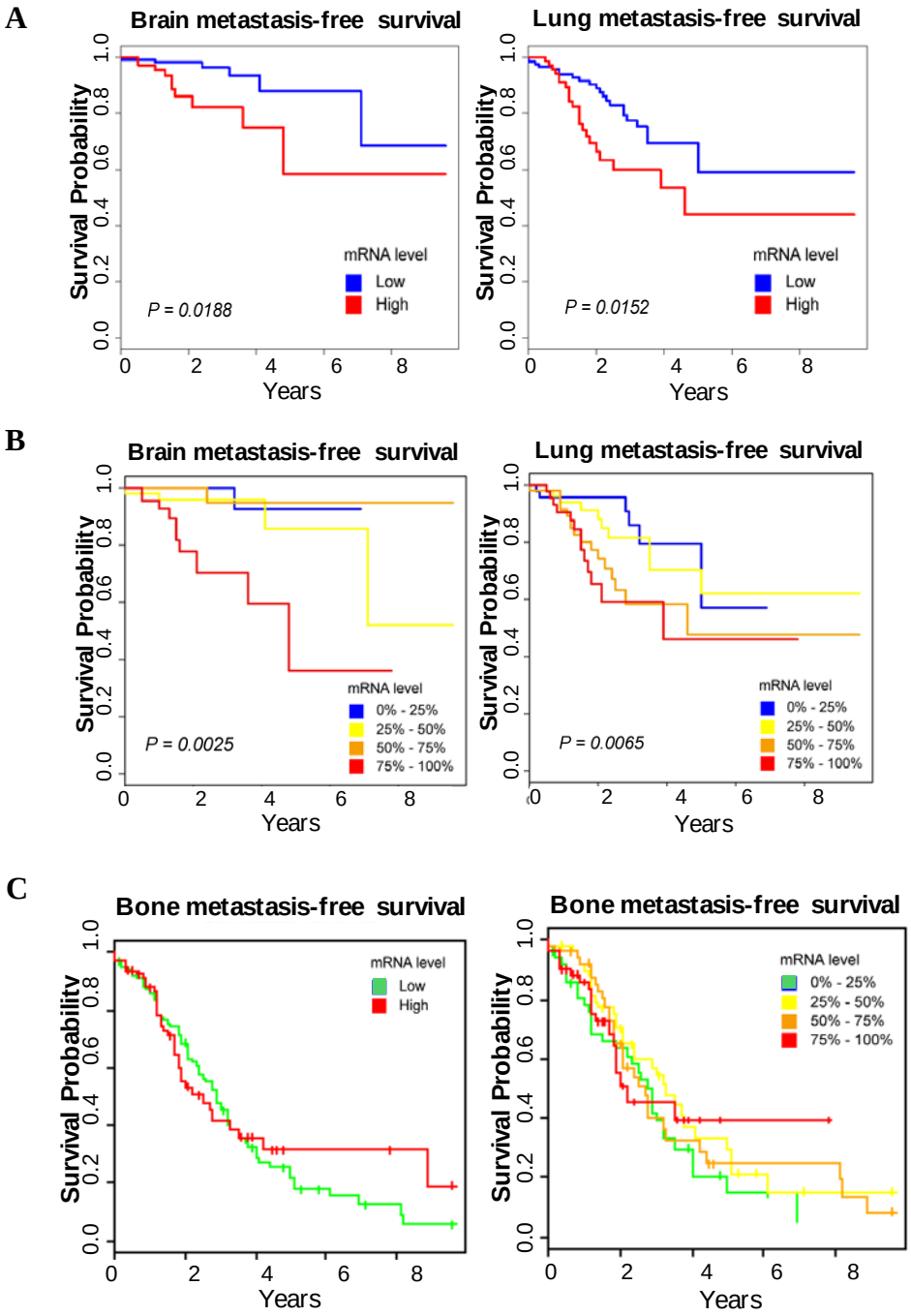

Figure 3.20 Kaplan-Meier analyses based on expression of 12 signature genes.

A. Kaplan-Meier analyses of disease-specific survival of breast cancer patients of Netherlands cohort. Patients were stratified by average expression of the 12 signature genes defined based on mean values.
B. Kaplan-Meier analyses of (A) by stratifying patients with quartiles partition.
C. Kaplan-Meier analyses of bone metastasis-free survival in breast cancer patients of Netherlands cohort whom were stratified by average expression of the 12 signature genes defined based on mean values or quartile partition.

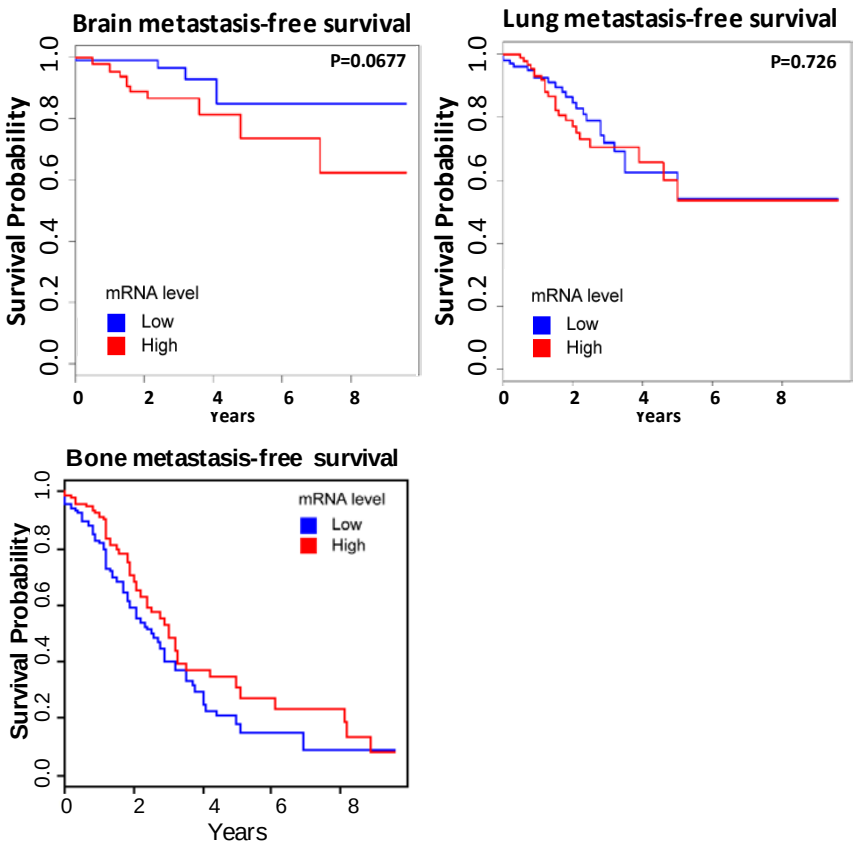

Figure 3.21 Kaplan-Meier analyses based on expression of EZH2/RelB independent RelA regulated genes

Kaplan-Meier analyses of disease-specific survival of breast cancer patients of Netherlands cohort. Patients were stratified by average expression of the EZH2/RelB-independent NF-κB regulated genes, defined based on mean values.

To validate the role of EZH2, RelA, and RelB in regulating the invasiveness of breast cancer cells, we performed transwell invasion assay using MB231 transient knocked-down with EZH2/RelA/RelB individual gene or in combination. We showed that each of the EZH2, RelA or RelB depletion reduced the invasive capacity of MB231 cells, and a combined knockdown gave rise to a more profound inhibition (Figure 3.22A). A similar result was also observed when we performed 3D matrigel assay (Figure 3.22B). 3D matrigel assay assesses the capability of cancer cells in conferring anchorage-independent growth, a characteristic of aggressive breast cancer. Overall, these results indicate that EZH2, RelA, and RelB can regulate the invasive and aggressive behaviors of BLBC cells through both overlapping and independent mechanisms.

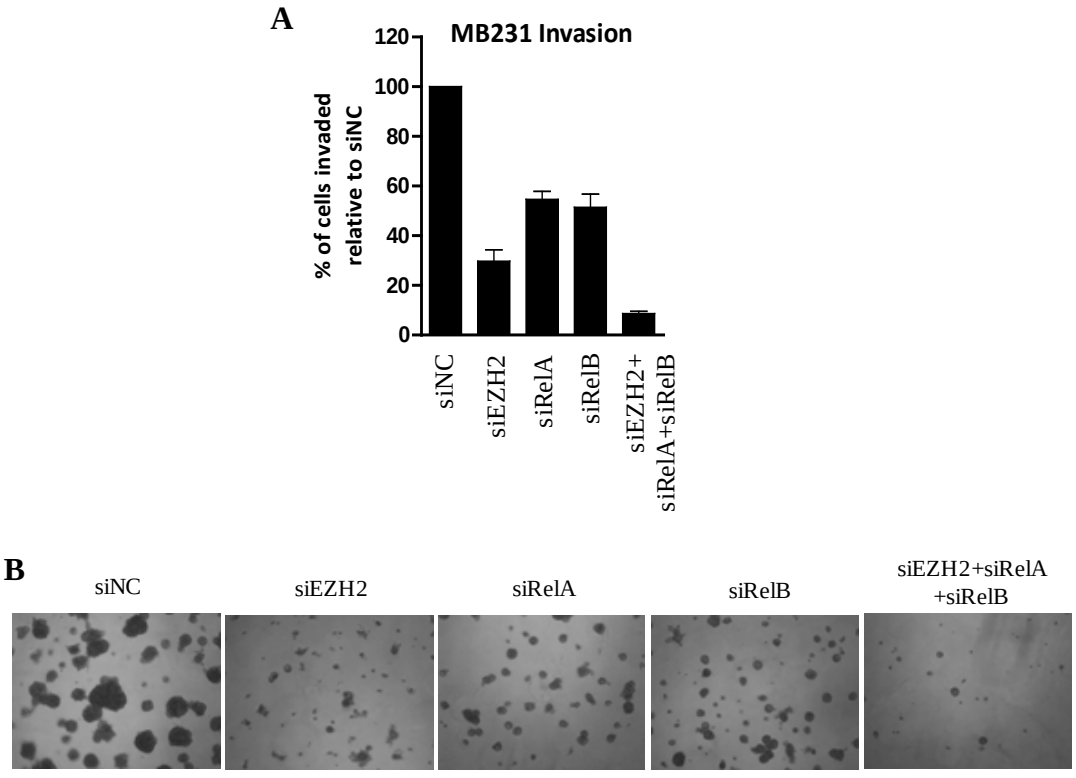

Figure 3.22 EZH2, RelA, and RelB depletion reduced invasiveness and aggressiveness of MB231.

- **A.** Transwell invasion assay was performed on EZH2, RelA, and/or RelB depleted MB231.
- **B.** 3D matrigel assay was performed on EZH2, RelA, and/or RelB depleted MB231. Phase-contrast images were taken under 40X magnification on the 8th day post seeding.

CHAPTER 4: EZH2 AND NF-KB CROSSTALK IN LUMINAL BREAST CANCER

4.1 EZH2 Negatively Regulates NF-κB Target Genes in ER Positive Luminal Breast Cancer Cells

Our lab has previously reported that EZH2 depletion in ER positive luminal MCF7 cells activates the expression of many inflammatory cytokines, including known NF-κB targets (Sun *et al.*, 2009). This discrepancy between luminal and basal-like breast cancer cell lines such as MB231 and BT549 suggests that EZH2 could acts differently in regulating NF-κB target gene expression, depending on the cellular context. ER has been known for long to repress NF-κB activity and target gene expression (Nakshatri *et al.*, 1997; Pratt *et al.*, 2003). In addition, ER has also been reported to repress RelB expression (Wang *et al.*, 2007) in MCF7 cells. Given that RelB plays essential roles in the formation and DNA binding activity of EZH2/RelA/RelB complex (refer to Chapter 3), the underexpression of RelB in luminal cells could lead to an altered function of EZH2 in regulating NF-κB targets. In agreement with the earlier findings, we found many NF-κB targets were highly expressed in ER negative MB231 cells but repressed in ER positive MCF7 cells. This pattern of expression was inversely correlated with the expression of *ER* and the two well-known ER co-factors *FOXA1* and *GATA3* in the two cell lines (Figure 4.1).

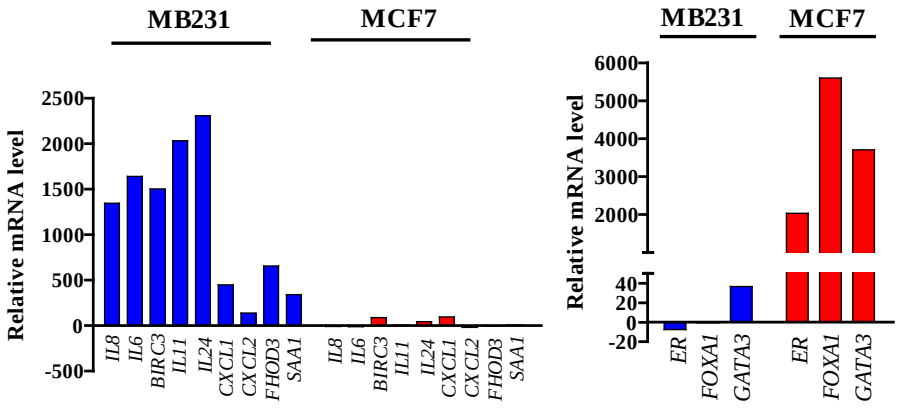

Figure 4.1 Inversely correlated expressions of NF-κB targets and ER-related genes in MB231 and MCF7.

Bar graphs showing the mRNA levels of NF-κB target genes (Left) and ER-associated genes (Right) in MB231 and MCF7. Raw values were extracted from a microarray gene expression profiling assay that was previously done in our lab.

Consistent with the previous study (Sun *et al*., 2009), the depletion of either EZH2 or ER in the ER-positive MCF7 and T47D breast cancer cells resulted in marked induction of *IL6* and *IL8* expression at both the basal level and after TNFα treatment (Figure 4.2, Top), validating the repressive roles of EZH2 and ER in regulating NF-κB target genes. To confirm that *IL6* and *IL8* were NF-κB targets in these two cell lines, knockdown of RelA was performed and as expected, their expressions were markedly abolished. It is worth to note that the depletion of EZH2 or ER did not affect RelA protein level (Figure 4.2, Bottom), suggesting that the negative regulation by these two factors might be direct.

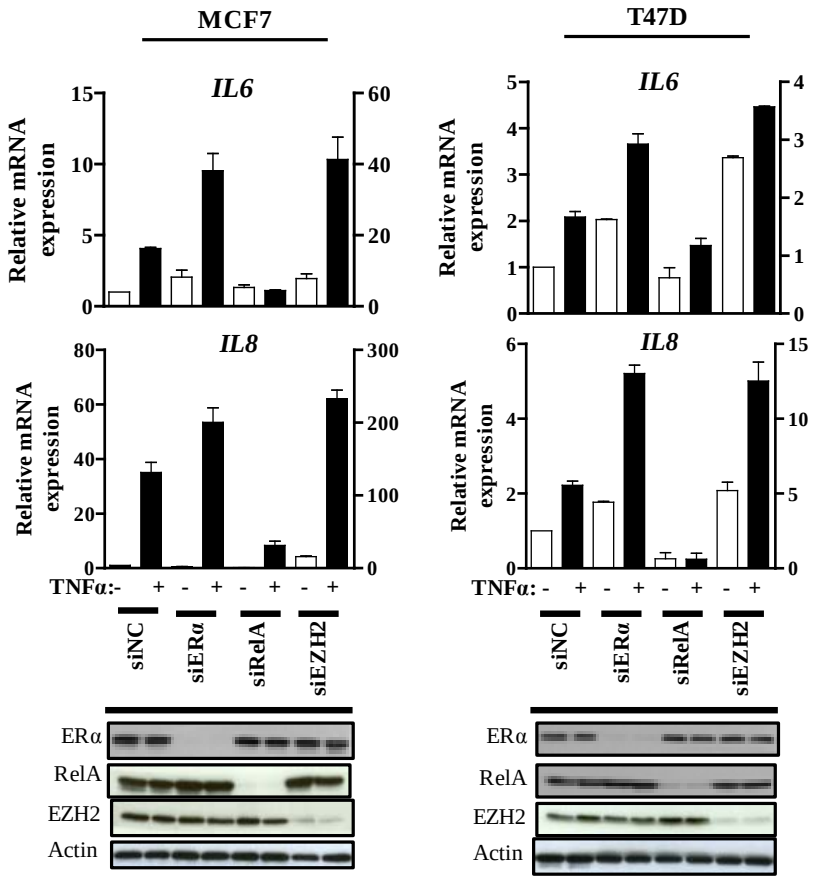

Figure 4.2 ER and EZH2 depletion enhanced IL6 and IL8 expressions.

qRT-PCR analysis of *IL6* and *IL8* expression in MCF7 (Left) and T47D (Right) upon ER, RelA, or EZH2 knockdown followed by TNFα treatment for 4 hours. Relative mRNA expression level after EZH2 knockdown was plotted on the right y-axis.

4.2 EZH2 interacts with ER and co-occupy NF-κB target genes promoter together with the enrichment of H3K27me3 mark

EZH2 has been previously shown to function as a corepressor in ER-mediated gene regulation in ER-positive breast cancer cells (Hwang *et al.*, 2008). In attempt to explore this potential role of EZH2 in MCF7 cells, we first conducted co-IP assay to detect the interaction between the PRC2 components, EZH2 and SuZ12, with ER in MCF7 cells. Indeed, both EZH2 and SuZ12 were detected in ER immunoprecipitates, and similarly, ER was detected in EZH2 immunoprecipitates (Figure 4.3). Importantly, in both immunoprecipitates, RelA and RelB were not detected. This indicates that PRC2 formed a complex with ER but not with RelA/RelB in MCF7 cells. Intriguingly, the PRC2-ER interaction was disrupted after TNFα-treatment. This suggests a possibility that ER may recruit PRC2 to promoters of NF-κB target genes for transcriptional repression but upon TNFα treatment, the repressive PRC2-ER complex might be disrupted and shattered, potentially due to the recruitment of NF-κB and its coactivators, resulting in the induction of the target genes expression.

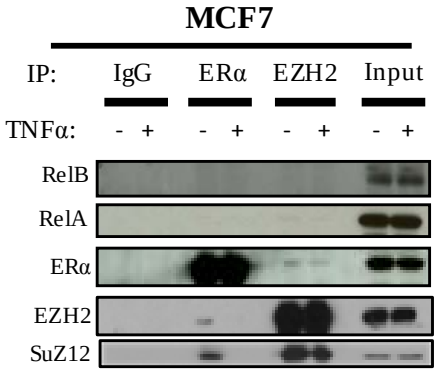

Figure 4.3 ER interacted with EZH2 and SuZ12 and dissociated in response to TNFα.

Co-IP of MCF7 cell lysates using indicated antibodies followed by Western Blot analysis.

To examine this speculation, we performed ChIP analysis in MCF7 cells. We detected marked enrichment of EZH2 and ER on *IL6* and *IL8* promoters, with concomitant high level of H3K27me3 but low level of RelA binding under unstimulated condition (Figure 4.4A). This observation is consistent with the reduced expression of *IL6* and *IL8*, which could be reversed by ER or EZH2 depletion in MCF7 cells (Figure 4.2). Moreover, following TNFα treatment over time, the amount of EZH2 and ER as well as H3K27me3 associated with *IL6* and *IL8* promoters dropped steadily, in contrast to the increased recruitment of RelA (Figure 4.4B). This change of promoter occupancies by these factors corresponded well to the strong induction of *IL6* and *IL8* (Figure 4.2). These observations support that ER co-recruitment of PRC2 to NF-κB target genes may account for their silenced expression in MCF7 cells under unstimulated condition. Under unstimulated condition, ER could recruit PRC2 complex to *IL6* and *IL8* promoter to catalyze H3K27 trimethylation, leading to subsequent suppression of the target genes. Under TNFα-stimulated condition, the recruitment of RelA transactivational complex, the concomitant reduction of PRC2 complex would cause a decrease in H3K27me3, thereby facilitating the induction of the target genes expression.

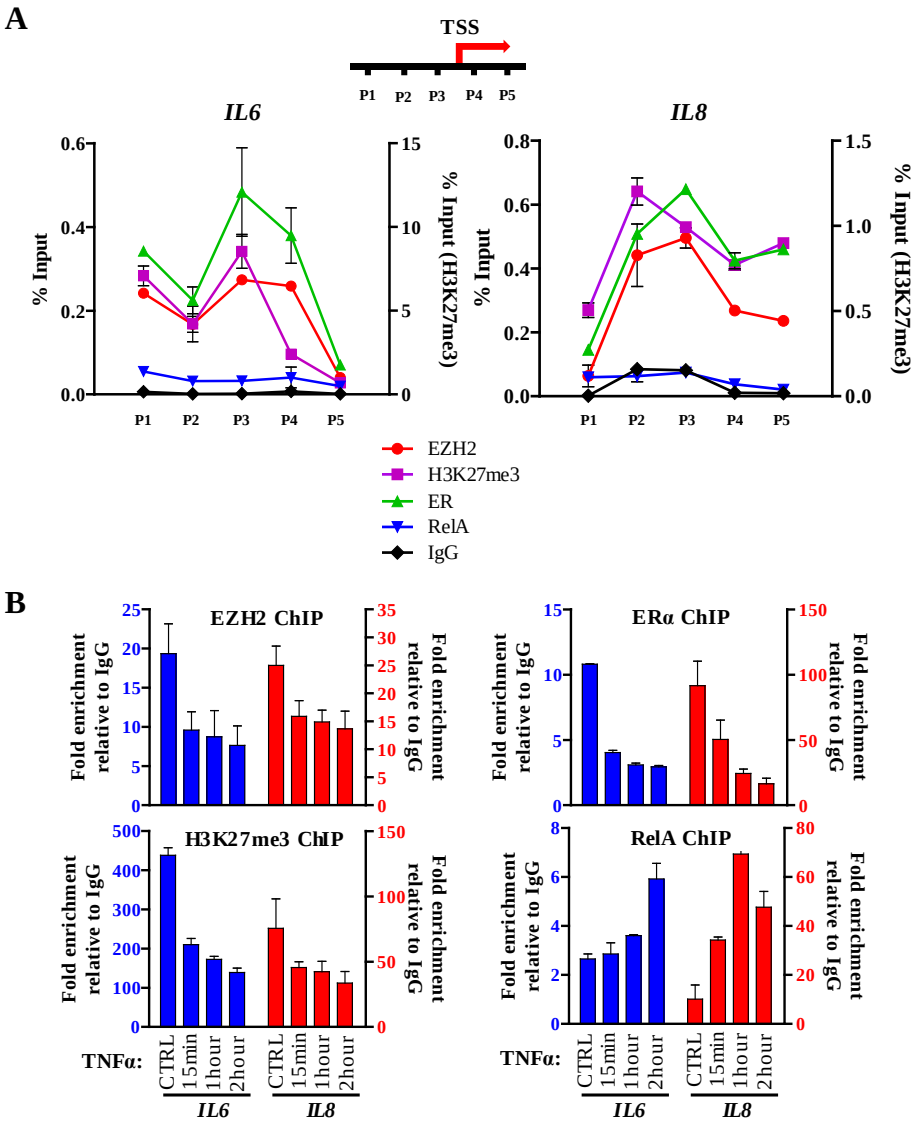

Figure 4.4 ER, EZH2, and H3K27me3 enrichment on IL6 and IL8 promoter reduced upon TNFα stimulation.

A. ChIP assay of EZH2, H3K27me3, ER, or RelA on the promoters of *IL6* and *IL8* in unstimulated MCF7 cells. Primer pairs spanning a 2.5 kb genomic region surrounding the TSS are indicated. P3 is located in the region within 500 bp upstream of TSS. Quantification of enrichment was determined as a percent of input DNAs.

B. ChIP assay of EZH2, H3K27me3, ER, or RelA on the promoters of *IL6* (P3) and *IL8* (P3) in MCF7 cells treated with TNFα for indicated times. Quantification of binding was represented as fold enrichment over IgG.

4.3 Ectopic RelB expression alters EZH2 regulation of NF-κB targets

One potential reason underlying the difference of EZH2 functions in modulating NF-κB target gene expression in BLBC and luminal breast cancer cells could be the underexpression of RelB. To investigate whether the restoration of RelB expression in luminal breast cancer cells could alter EZH2 function, we stably overexpressed RelB in MCF7. Excitingly, ectopic RelB overexpression in MCF7 cells results in strong induction of *IL6*, which could be reversed by EZH2 knockdown (Figure 4.5). In contrast, RelB overexpression in MCF7 cells only resulted in a modest induction of *IL8* and EZH2 knockdown caused further elevated its expression. These results partially recapitulated the phenomena observed in the BLBC cell line, MB231, in two ways: (i) RelB governed the expression of *IL6* but not *IL8*; and (ii) EZH2 could positively regulate *IL6* expression in the presence of RelB. Although *IL8* appeared to remain negatively regulated by EZH2 upon RelB overexpression, this could be reasoned by the existence of PRC2-dependent ER-mediated repression of the gene, which is independent of RelB. Taken together, these results highlight a crucial role of RelB in selective activation of NF-κB targets in a EZH2-dependent manner.

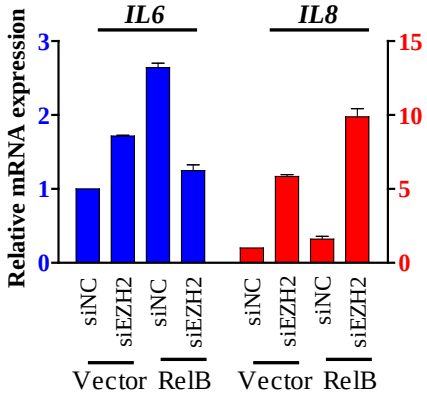

Figure 4.5 Ectopic RelB expression in MCF7 reversed EZH2 regulation of IL6 expression.

RT-PCR analysis of *IL6* and *IL8* mRNAs in MCF7 cells with stable overexpression of vector or RelB with or without EZH2 siRNA treatment.

CHAPTER 5: CONCLUSIONS AND PROPOSED MODEL

Conclusions and Proposed Model

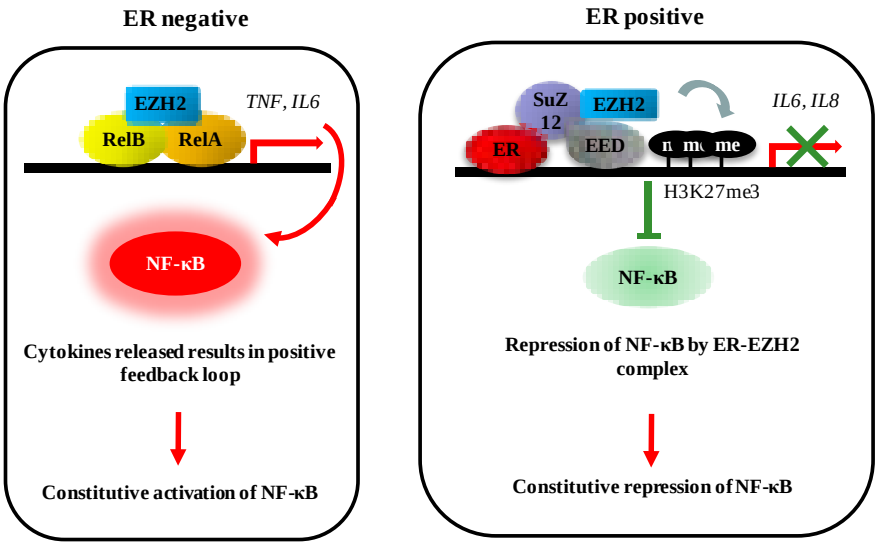

Figure 5.1 A proposed model of context-dependent EZH2 modulation of NF-κB pathway in breast cancer.

In ER-negative basal-like breast cancer cells, EZH2 acts as a co-activator of RelA and RelB to promote the expression of NF-κB target genes such as *TNF* and *IL6*, which in turn activates NF-κB signaling through a positive feedback, leading to constitutive activation of NF-κB target gene expression. In ER-positive breast cancer cells, ER recruits PRC2 complex to the promoter of NF-κB target genes, leading to epigenetic silencing of NF-κB target genes.

Based on the collective results of Chapter 3 and Chapter 4, we proposed a model for EZH2 regulation of NF-κB target gene expression in ER positive and ER negative breast cancer cells that is summarized in Figure 5.1. In ER negative breast cancer, overexpression of both EZH2 and RelB forms a complex with RelA to confer constitutive activation of NF-κB target gene expression. On the other hand, in ER positive breast cancer cells where RelB expression is repressed, ER recruits PRC2 to NF-κB target gene promoters and function as a canonical epigenetic repressor. The differential expression of ER and RelB in the two contexts may be crucial for switching the role for EZH2 in regulating NF-κB target gene expression. Based on this model, we highlighted that EZH2 could play opposing roles in regulating NF-κB network through distinct mechanisms in a cellular context-specific dependent manner.

CHAPTER 6: DISCUSSION

6.1 New mode of NF-κB Constitutive activation in Aggressive Breast Cancers

As described in Section 1.3.2.2, several mechanisms have been proposed to account for the constitutive activation of NF-κB signaling in cancers, particularly in leukemia. For instance, Rel was found to be amplified in B-cell lymphoma, which led to hyperactivation of target genes in canonical NF-κB pathway. Besides, shorten IκB half-life and IκB mutation were observed in B-cell lymphoma and Hodgkin lymphoma, respectively, which released RelA from inhibition, leading to constitutive activation of canonical NF-κB pathway.

However, in breast cancer, mutations or genomic amplication of the components in the NF-κB pathway are uncommon. In search for the potential mechanisms conferring constitutive NF-κB activation, two independent studies led by Struhl K (Iliopoulos *et al*., 2009) and Luo JL (Rokavec *et al*., 2012) reported a similar conclusion: IL6 expression induced by a transient oncogenic "kick" was sufficient to drive the feed forward response of NF-κB pathway, leading to its constitutive activation (Figure 6.1). Specifically, both of these studies adopted MCF10A, an immortalized mammary epithelial cell line, as the model system and triggered the cells with transient SRC activation or MCP1 derived from monocyte conditioned medium. Consequently, induction of IL6 and concomitant suppression of microRNA, Let-7 or *miR200c*, would maintain the positive feedforward loop of NF-κB pathway activity. The self-sustainability of this loop was evident by the continuation of the pathway even after the removal of the activation triggers, SRC or MCP1 in these cases.

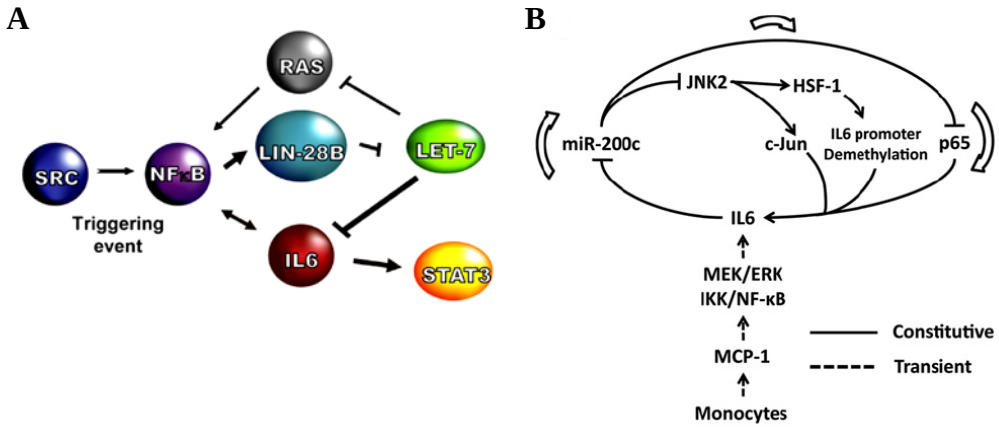

Figure 6.1 Two proposed models of constitutive activation of NF-κB in breast cancer.

A. Model proposed by Struhl K.
B. Model proposed by Luo JL

However, the findings of these two studies could not fully explain why constitutive activation of NF-κB is observed more profoundly in ER-negative BLBC compared to ER-positive luminal breast cancer. If transient activation of SRC or MCP1 is adequate to allow constitutive activation of NF-κB pathway, the chances of getting constitutively activated should be similar in BLBC and luminal breast cancer as the SRC and MEK pathways were activated in both cancer subtypes (Acosta *et al*., 2003; Rakha *et al*., 2008). Thus, one can speculate that additional pre-existing factors are required to be present in BLBC for sustaining the positive feedforward loop.

Based on our study, we put forward a model that supports this notion. That is, the overexpression of RelB with a concurrent absence of ER in BLBC might predispose activation of NF-κB by EZH2. Low expression of ER could be the cause of an enhanced expression of RelB in BLBC. Consequently, high level of RelB enhances the DNA binding of RelA and facilitates transactivation of NF-κB target genes together with EZH2. By regulating NF-κB pathway at the transcription factor level, constitutive activation of NF-κB could be achieved by circumventing the negative feedback mechanisms of the pathway such as the elevation of IκB production.

6.2 RelA and RelB Conundrums

Mechanistically, we show that EZH2 promotes NF-κB binding to a set of target gene promoters possibly through direct physical interaction with RelA and RelB in BLBC cells. In particular, RelB is shown to play a crucial role in this regulation. Although non-canonical NF-κB pathway mediated by RelB has been generally considered to be different from RelA-mediated canonical pathway and governs distinct categories of target gene expression in non-malignant models (Bonizzi *et al*., 2004), it was reported that RelB could recognize a more diversed range of κB sequences including those DNA motif bound by RelA (Fusco *et al*., 2009). In this study, we clearly see a significant set of NF-κB targets genes co-regulated by both RelA and RelB in BLBC cells, many of which were known to be regulated by RelA including *IL6*, *TNF*, *SAA1*, and *PTGS2*. This indicates an oncogenic role of RelB in co-regulating RelA targets in some malignancies and is consistent with a recent report that inhibition of RelB in prostate cancer could attenuate the canonical NF-κB target gene expression, resulting in decreased tumorigenicity (Xu *et al*., 2009).

RelA:RelB interaction was first demonstrated by Marienfeld *et al.* using whole cell extracts of several T- and B-cell lines (Marienfeld *et al*., 2003). In their study, they found that the dimerization of RelA and RelB resulted in the reduction of RelA DNA binding affinity, demonstrated by Electrophoretic Mobility Shift Assay (EMSA) using DNA probe with a single κB sequence. However, this assay was limited by its inability to determine the binding affinity of RelA:RelB complex to other variants of κB motifs. Possibly, while the binding affinity of RelA was decreased in that specific κB sequence adopted in the EMSA experiment, the affinity could be induced in other potential binding sites in the genome. As RelB was reported to have a more diverse recognition of κB sequences, it is likely that by binding to RelB, RelA could obtain a higher affinity to these alternative sites to trigger gene transcription. In our study, we clearly observed that in the absence of RelB, RelA binding to

the promoter of *TNF* and *IL6* drop drastically, indicating an enhancing role of RelB on RelA DNA binding at these regions. Conversely, depletion of RelB had minimal impact on RelA binding on *IL8* and *BIRC3* promoters, suggesting that RelB only affect RelA DNA binding at certain regions in the genome. It would be interesting to determine whether the cis regulatory elements of the subset of genes co-regulated by RelA and RelB has distinct DNA motif sequences compared to the subset of genes regulated mainly by RelA. To achieve this, genome-wide ChIP sequencing assay of RelA and RelB binding in BLBC cells would be necessary. It is worth mentioning that the co-regulated genesets found in this study might be specific to BLBC cells, in which RelB is aberrantly expressed and both RelA and RelB have essential roles in promoting cancer cell invasiveness.

6.3 Antagonism between EZH2/ER and NF-κB pathway

Intriguingly, EZH2 functions oppositely in ER positive breast cancer cells. We have previously shown that EZH2 could negatively regulate inflammatory gene network in ER positive and non-invasive breast cancer MCF7 cells (Sun *et al*., 2009). This discrepancy may arise from the heterogeneous molecular profiles embedded in the two different subtypes of breast cancers, in particular in relation to the ER status. It is well established that ER is inhibitory to both RelB and NF-κB signaling in breast cancers and that NF-κB targets are significantly elevated in ER negative breast tumors (Freund *et al*., 2003; Gamba *et al*.; Gionet *et al*., 2009; Kalaitzidis and Gilmore, 2005). However, the mechanism underlying this inhibition might not be necessary through inhibiting NF-κB DNA binding capacity (Liu *et al*., 2005; Ray *et al*., 1997). Consistent with this notion, we show in this study that ER recruits PRC2 to enforce a repressive chromatin modification at NF-κB target genes, thus providing an alternative mechanism to silence NF-κB target gene expression.

Based on our findings, ER-EZH2 occupancy at NF-κB target gene promoters is mutually exclusive with the occupancy of RelA. Upon TNFα stimulation, ER-PRC2 repressor complex as well as H3K27me3 mark was reduced concomitantly with an increased in RelA association with NF-κB targets. TNFα treatment is known to increase RelA association with histone acetylase CBP/p300 at its target promoters (Gerritsen *et al*., 1997; Perkins *et al*., 1997). We postulate that the increased histone acetylation may confer a more open chromatin architecture that is not favourable for PRC2 recruitment upon TNFα treatment. Besides, TNFα stimulation of RelA led to the decrease of H3K27me3 mark as early as 15 minutes, suggesting that certain machinery could be turned on rapidly to actively remove the repressive mark. Interestingly, it was reported that induction of NF-κB pathway could upregulate the expression of Jumonji D3 (JMJD3), which was found to have demethylation activity specifically at H3K27 (De Santa *et al*., 2007).

6.4 Context-specific mode of NF-κB pathway regulation by EZH2 and its Clinical Implications

We have demonstrated that in a cellular context characterized by presence of RelB and absence of ER, EZH2 could positively regulate NF-κB target genes. Conversely, in the presence of ER and absence of RelB, EZH2 could collaborate with ER to suppress NF-κB target genes. Therefore, the counterbalance between ER and RelB expression in ER positive and ER negative breast cancer cells appears to be crucial in determining EZH2 role as a repressor or an activator of NF-κB targets in breast cancer cells.

Interestingly, it was reported that tamoxifen (ER antagonist) response is more favourable in tumors with low EZH2 expression (Reijm *et al*., 2011). If we apply our proposed model in this clinical observation, it is possible that in response to long-term inhibition of ER by tamoxifen, RelB could be upregulated, leading to the constitutive activation of NF-κB target genes by RelA/RelB when EZH2 is also expressed at a higher level. Hence, tamoxifen treatment could be beneficial to those patients with lower EZH2 expression as they exhibit lower propensity towards the EZH2-mediated constitutive activation loop of NF-κB pathway.

In addition, it was observed that patients with low EZH2 expression in inflammatory breast cancer would confer better prognosis (Gong *et al*., 2011). Albeit EZH2 was implicated in the activation of NF-κB through suppressing DAB3IP, it could not explain its role in constitutive activation of NF-κB in inflammatory breast cancer. Alternatively, by applying our proposed model in this scenario, this observation may imply that high expression of EZH2 in inflammatory breast cancer could result in constitutive activation of NF-κB signaling through its SET-domain independent activity, which cold be detrimental to the patients.

6.5 EZH2 acts more than a methyltransferase in oncogenic progression

In 1990s, EZH2 was first discovered to play a critical role in maintaining stem cell pluripotency by suppressing the expression of differentiation genes (Laible *et al*., 1997). In 2000s, research on EZH2 was altered to focus on the role of PRC2 in promoting tumorigenesis of multiple types of malignancies including leukemia, prostate and breast cancers (Sauvageau and Sauvageau, 2010). The oncogenic roles of EZH2 are mainly attributed to its transcriptional repression activity on various tumour suppressor genes including CDKN1C, CDH1, and Bim (Cao *et al*., 2008; Wu *et al*., 2010; Yang *et al*., 2009a). This gene silencing function of EZH2 is dependent on its enzymatic activity in catalyzing the trimethylation at Histone 3 Lysine 27 (H3K27me3) (Cao and Zhang, 2004a). Studies have demonstrated that in order for EZH2 to function as a histone methyltransferase, it has to associate physically with suppressor of zeste 12 (SuZ12) and embryonic ectoderm development (EED) to form the core complex of Polycomb Repression Complex 2 (PRC2) (Cao and Zhang, 2004b).

Intriguingly, despite of the frequent overexpression of EZH2 in a range of malignancies, its interacting partners of the core PRC2 complex, in particularly EED was not found to be substantially overexpressed in cancers (Chase and Cross, 2011). On the contrary, homozygous hypomorphic or heterozygous null mutations of EED in mice was demonstrated to promote relapse of lymphoma (Sauvageau and Sauvageau, 2010). One of the possibilities is that PRC2 complex could function as a tumor suppressor. However, this phenomenon was not demonstrated thus far. Another possibility is that in the absence of EED, the stoichiometry of PRC2 complex would be imbalanced and resulting in the excessive EZH2 to function independently of PRC2 complex. This gain of function of EZH2 independent of PRC2 would confer oncogenic propensity. Recent studies have discovered recurrent missense or nonsense mutations at EZH2 gene locus that abolish its catalytic activity in myeloid disorders (Ernst *et*

al., 2010; Makishima *et al*., 2010; Score *et al*., 2012). Such mutations were also shown to be associated with poor prognosis. Again, it is possible that the catalytic incompetent EZH2 mutant could also exhibit gain-of-function activity and confer oncogenesis. This could be exemplified by Shi *et al.* and our study demonstrating that EZH2 could serve as a transcriptional activator of two oncogenic signaling pathways, ER/β-catenin and NF-κB, which would further contribute to tumor progression (Lee *et al*., 2011; Shi *et al*., 2007).

Although the transcriptional repressive function of EZH2 was long known to be responsible for tumor progression, the critical tumor stage at which H3K27 trimethylation contributes to oncogenesis is largely unknown. A recent study reported that H3K27 trimethylation occurs as an early epigenetic event in hepatocellular carcinogenesis by silencing $p16^{INK4a}$, and this repressive mark would then be replaced by H3K9 dimethylation during the later development (Yao *et al*., 2010). An independent study showed that H3K9 dimethylation preceeds DNA methylation and histone deacetylation on $p16^{INK4a}$ locus and the latter epigenetic marks serve to maintain the gene silencing. Besides, PRC2 complex is known to recruit histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) during the course of transcriptional repression (Bachman *et al*., 2003). Paradoxically, researchers have found DNA methylation to be independent of H3K27me3 and the overlap between DNA methylated and H3K27 trimethylated regions is modest (Kondo *et al*., 2008). One possible explanation is that when PRC2 catalyzes H3K27me3 during early stages of oncogenesis, the complex would recruit DNMTs that would catalyze DNA methylation for long term suppression. While the H3K27me3 marks would subsequently being erased by other histone modifying enzymes over time. These findings suggest that epigenetic modification appears to be a dynamic event during oncogenic progression. PRC2-mediated H3K27me3 could be an early event of tumor progression and this suppressive mark could be subsequently substituted by other stable epigenetic repressive mechanisms (e.g., DNA methylation) at the later stage of oncogenesis.

Given that PRC2-mediated transcriptional repression could potentially be replaced by other long term repressive mechanisms, would this activity still be substantial and essential as the tumor progresses? Wei *et al* demonstrated by immunohistochemical staining showing that H3K27me3 level decreases as the tumor develops to the more advance stage (Wei *et al*., 2008). In this study, it was shown that breast, ovarian, and pancreatic tumors with larger size, higher tumor grade, and more lymph node metastasis tend to have lower H3K27me3 level, albeit higher level of EZH2 was found to be associated with the more advance tumors. Patients with lower H3K27me3 and higher EZH2 level were therefore associated with poor prognosis. This seems to suggest that although EZH2-mediated H3K27me3 modification became less substantial as the tumor progressed, high level of EZH2 remained essential in advance tumors. This implies that EZH2 could exert oncogenic influence through means other than its H3K27-trimethylating catalytic activity for promoting cancer progression. We have reported that EZH2 could act as a transcriptional activator independent of its methyltransferase activity, promoting NF-κB signaling in the more aggressive ER-negative breast cancer and confers poor clinical outcome. In agreement with our findings, Wei *et al* also reported that EZH2 is more overexpressed in ER-negative breast cancer although H3K27me3 level is lower in this cancer subtype (Wei *et al*., 2008).

6.6 Significance of Study

Both EZH2 and NF- B contribute to aggressive breast cancer, yet existence of a functional crosstalk between these two factors in breast cancer is still unclear. Here, we uncover an unexpected role of EZH2 in inducing NF-κB target gene expression in ER-negative basal-like breast cancer cells. This function of EZH2 is independent of its histone methyltransferase activity but requires the physical interaction with RelA/RelB. Intriguingly, EZH2 acts oppositely in repressing NF-κB targets in ER-positive luminal breast cancer cells by interacting with ER and directing repressive H3K27 trimethylation. Thus, EZH2 function as a double-facet molecule in breast cancers, functioning either as a transcriptional activator or repressor of NF-κB targets, in a cell context-dependent manner.

Targeting of NF-κB in cancer therapeutics has been jeopardized by its essential roles in maintaining normal physiological homeostasis (Baud and Karin, 2009). Hence, it would be beneficial to target certain pathways that are specifically activated in cancers. Our findings of EZH2 transactivation function on NF-κB pathway could be one such example. Our data suggest that this transactivation property require at least two crucial factors: (i) higher expression of RelB and (ii) aberrant overexpression of EZH2. These criteria should not be fulfilled in healthy cells under normal circumstances, but were observed frequently in aggressive ER-negative breast cancer cells. Hence, drugs targeting this oncogenic complex would be specific to this subtype of breast cancer.

In the development of EZH2 targeted therapy, research effort was focused on small molecule inhibitors of EZH2 enzymatic activity. 3-Deazaneplanocin A (DZNep) is one of S-adenosylhomocysteine (AdoHcy) hydrolase inhibitors that was shown to be able to deplete PRC2 complex and hence exert antitumor activity in various types of cancers (Tan *et al*., 2007). However, reports have shown that the specificity of DZNep is suboptimal and it could cause a reduction of global histone methylation (Miranda *et al*., 2009). This indicates the need for more specific inhibitors. Nevertheless, given that EZH2 could act independently of its

methyltransferase activity in promoting aggressive phenotypes in ER-negative breast cancer cells, the effectiveness of the small molecule inhibitors targeting EZH2 methyltransferase activity could be limited. Therefore, novel targeting approaches such as interfering with EZH2 transactivation function may be considered for the treatment of advance tumors that have high EZH2 level but low H3K27me3. It is worth to mention that patient stratification would be a necessary procedure prior to implementing these EZH2 targeted strategies. This is especially so as EZH2 could repress NF-κB signalling in ER-positive breast cancer cells, inhibiting its methyltransferase activity in this breast cancer subtype may result in undesirable activation of NF-κB network. The 12 signature genes identified from the breast cancer cell line panel in this study could potentially be adopted for predicting BLBC patient subsets that would respond to inhibitors targeting EZH2-NF-κB interactions.

6.7 Future Prospects

In this study, we have detected the physical interaction between EZH2, RelA, and RelB, which enhanced the aggressiveness of BLBC cells. The next immediate question would be the identification of protein domains in EZH2, RelA, and RelB responsible for the interactions that stabilized this complex. This is a necessary experiment before moving on to the development of interfering peptide inhibitors specific to the EZH2-NF-κB interactions. Thus far, most peptide inhibitors that were approved in clinical trials usually target cell-surface receptor as a result of serious challenges associated with peptide delivery into the cells. Given that many natural products were found to have activity interfering protein-protein interactions (as mentioned in section 1.3.2.3), screening of natural products could be a good starting point to search for compounds interfering EZH2/RelA/RelB interactions.

Based on our data, we found that EZH2 could also function as a corepressor of ER in ER-positive breast cancer cells to suppress NF-κB target genes. It would be interesting to investigate if such transcriptional collaboration with EZH2 could also be observed for other hormone nuclear receptor in other types of cancers, for instance ovarian and prostate cancers. Indeed, recent reports have demonstrated that EZH2 could act as a corepressor of AR in prostate cancer (Chng *et al*., 2012; Yu *et al*., 2010). Therefore, it might be worthwhile to establish the role of EZH2 with respect to different types of cancers so as to assess the feasibility of EZH2 targeted therapy in different cancer types.

APPENDICES

Appendix 1: Quantitative Realtime PCR of cDNA primer sequences:

Gene	Forward primer	Reverse primer
18S	CGAACGTCTGCCCTATCAACTT	ACCCGTGGTCACCATGGTA
IL8	GAGTGATTGAGAGTGGACCACACT	AGACAGAGCTCTCTTCCATCAGAAA
IL6	GTACATCCTCGACGGCATCTC	GCTGCTTTCACACATGTTACTCTTG
TNFα	CCCAGGCAGTCAGATCATCTTC	GGTTTGCTACAACATGGGCTACA
BIRC3	GGCTTGAGGTGTTGGGAATC	ACTCACACCTTGGAAACCACTTG

Appendix 2: Quantitative Realtime PCR of ChIP primer sequences:

Gene	MB231	MCF7	Forward primer	Reverse primer
IL6	**P1**	**-**	TAGGTGCTACCTCTGGGAAAAGG	AGGGCATGCAGGGAAAAGT
	P2	**P1**	AGGCGGGTCCTGAAATGTT	GACCTCTGTTGGGCATTTACTCA
	P3	**P2**	TGGAGACGCCTTGAAGTAACTG	GTGAGCGGCTGTTGTAGAACTG
	P4	**P3**	GTCTGAGGCTCATTCTGCCC	AGAGCTTCTCTTTCGTTCCCG
	P5	**P4**	CCAGTACCCCCAGGAGAAGAT	AAGAGGTGAGTGGCTGTCTGTGT
	-	**P5**	AACTCAATGGCTAGGATTCCTCAA	CCACTTTGGTGGGCTCTGA
TNF	**P1**	**P1**	AAATCAGTCAGTGGCCCAGAA	CCCTCACACTCCCCATCCT
	P2	**P2**	CGCCACATCCCCTGACA	CGTGGGTCAGTATGTGAGAGGAA
	P3	**P3**	AGAGCTGTTGAATGCCTGGAA	CTGGCCTGCGCTCTTAGC
	P4	**P4**	CTCGAACCCCGAGTGACAA	AAGACACATCCTCAGAGCTCTTACC
	P5	**P5**	TGCTCCTCACCCACACCAT	GGAGGTTGACCTTGGTCTGGTA
IL8	**P1**	**P1**	GCCACCTTTTTATGATTTGTTGAA	CCTCTGAGGACCCATGATCACT
	P2	**P2**	GTGCTGTTCTCTTTCATCTTCCTCTA	CAGTTGGAGCAAGGCATTGA
	P3	**P3**	TGGGCCATCAGTTGCAAA	ACTTATGCACCCTCATCTTTTCATT
	P4	**P4**	AAACCACCGGAAGGAACCA	CACGGCCAGCTTGGAAGT
	P5	**P5**	ACTAACTGAGGTCAAGGGCTAGGA	GAGACTATGGAAGGCATCATGTTTC
BIRC3	**P1**	**P1**	AATGGGCAAGGGAATGCA	GCAGGCCTTACACATTTTTGATT
	P2	**P2**	GGGAAATATGGCAGTGCAATTAG	TTCTGAGTTGCAGTGCCATTCT
	P3	**P3**	TCCCCGAGTGGGTTTGC	AGCGGTAATAACCACACACACTTC
	P4	**P4**	TTGTGAAGTTGTGGCATTTTGAT	CCTGCAAAGGCCCAGTGAT
	P5	**P5**	GGAAACTGAGGCTCTTGGAGATT	CAAGCAGTCCGGTAACCAGAA

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List of Publications

1. **Shuet Theng Lee**, Zhimei Li, Zhenlong Wu, Meiyee Aau, Peiyong Guan, R.K. Murthy Karuturi, Yih Cherng Liou and Qiang Yu. "Context-Specific Regulation of NF-kB Target Gene Expression by EZH2 in Breast Cancers". **Molecular Cell** , 43, 798–810, 2011.

2. Jing Tan, Zhimei Li, Puay Leng Lee, Peiyong Guan, Mei Yee Aau, **Shuet Theng Lee**, Min Feng, Cheryl Zhihui Lim, Eric Yong Jing Lee, Zhen Ning Wee, Swea Ling Khaw, Yusuke Yamamoto, Yaw Chyn Lim, Frank McKeon, Wa Xian, Bing Lim, R. K. Murthy Karuturi and Qiang Yu. "PDK1 Signaling Towards PLK1-Myc Activation Confers Oncogenic Transformation, Tumor Initiating Cell Activation and Resistance to mTOR-targeted Therapy". **Cancer Cell**, in revision.

3. Zhenlong Wu, **Shuet Theng Lee**, Yuanyuan Qiao, Zhimei Li, Puay Leng Lee, Yong Jing Lee, Xia Jiang, Jing Tan, Meiyee Aau, Cheryl Zhi Hui Lim, and Qiang Yu. EZH2 regulates cancer cell fate decision in response to DNA damage. **Cell Death Differ** , 2011 Nov;18(11):1771-9

4. Qiao Y, Jiang X, **Lee ST**, Karuturi RK, Hooi SC, Qiang YU "FOXQ1 regulates epithelial-mesenchymal transition in human cancers." **Cancer Res**, 2011 Apr 15;71(8):3076-86. Epub 2011 Feb 23.

5. Conference paper: "Epigenetic regulation of breast cancer immunity by EZH2." **ShuetTheng Lee**, Feng Sun, Eli Chan, Zhenlong Wu, Xiaojing Yang, Qiang Yu. *Molecular Basis for Chromatin Structure and Regulation,* January 17- 22, 2010, Keystone Symposia Conference at the Sagebrush Inn and Conference Center, Taos, New Mexico, USA.

6. Conference paper: "Different modes of regulation on inflammatory network by EZH2 in basal and luminal breast cancers." **ShuetTheng Lee**, Qiang Yu. *Stem Cells, Cancer and Metastasis*, March 6-11, 2011, Keystone Symposia Conference at the Keystone Resort, Keystone, Colorado, USA.

7. Conference paper: "Context-Specific Regulation of NF-kB Target Gene Expression by EZH2 in Breast Cancers". **Shuet Theng Lee**, Zhimei Li, Zhenlong Wu, Meiyee Aau, Peiyong Guan, R.K. Murthy Karuturi, Yih Cherng Liou and Qiang Yu. *Cellular Signaling and Molecular Medicine*, May 25-29, 2012, EMBO conference at Cavtat, Dubrovnik, Croatia.